Isolation, Structure Determination and Biological Activity Assessment of Secondary Metabolites from Marine-derived Fungi

Von der Gemeinsamen Naturwissenschaftlichen Fakultät
der Technischen Universität Carolo-Wilhelmina
zu Braunschweig
zur Erlangung des Grades einer
Doktorin der Naturwissenschaften
(Dr. rer. nat.)
genehmigte
D i s s e r t a t i o n

von Claudia Osterhage
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1. Referentin: Prof. Dr. G. M. König
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eingereicht am: 23.10.2000
mündliche Prüfung (Disputation) am: 19.03.2001

2001
(Druckjahr)

Vorveröffentlichungen der Dissertation / In Advance Publications of the Dissertation

Teilergebnisse aus dieser Arbeit wurden mit Genehmigung der Gemeinsamen Naturwissenschaftlichen Fakultät, vertreten durch die Mentorin/Betreuerin der Arbeit, in folgenden Beiträgen vorab veröffentlicht:

Parts of this study have been published in advance after permission of the Gemeinsame Naturwissenschaftliche Fakultät, represented by the supervisor of this study:

Publikationen / Research Papers

Osterhage, C., Schwibbe, M., König, G. M., and Wright, A. D.; Differences between Marine and Terrestrial *Phoma* species as Determined by HPLC-DAD and HPLC-MS. *Phytochem. Anal.* **11**, 1-7 (2000).

Osterhage, C., Kaminsky, R., König, G. M., and Wright, A. D.; Ascosalipyrrolone A, an Antimicrobial Alkaloid, from the Obligate Marine Fungus *Ascochyta salicorniae*. *J. Org. Chem.*, **65**, 6412-6417 (2000).

Osterhage, C., Höller, U., König, G. M., and Wright, A. D.; Drechslerines A-J from the algicolous fungus *Drechslera dematioidea*. *J. Nat. Prod.*, submitted.

Osterhage, C., Kaminsky, R., König, G. M., and Wright, A. D.; Epicoccamide, a Novel Secondary Metabolite from a Jellyfish-derived Culture of *Epicoccum purpurascens*. *J. Nat. Prod.*, submitted.

Osterhage, C., König, G. M., and Wright, A. D.; 5-Hydroxyramulosin, a New Natural Product obtained from *Phoma tropica*, a Marine-derived Fungus from the Alga *Fucus spiralis*. *Bull. Univ. Azores*, submitted.

Tagungsbeiträge / Research Presentations

C. Wegner, G. M. König, A. D. Wright; HPLC-MS and HPLC-DAD as tools to compare extracts from fungi of marine and terrestrial origin. Poster presented at the 1st Euroconference on Marine Natural Products, Athens, Greece, 2-6 November 1997.

C. Wegner, A. D. Wright, G. M. König; Fungi from marine algae as a source of new natural products with therapeutic potential. Poster presented at the fourth European Congress of Pharmaceutical Sciences, Milan, Italy, 11-13 September 1998.

C. Wegner, U. Höller, G. M. König, A. D. Wright; New bioactive compounds from the marine fungi *Ascochyta salicorniae* and *Ulocladium botrytis*. Poster presented at 2000 Years of Natural Products Research - Past, Present and Future, Amsterdam, Netherlands, 26-30 July 1999.

C. Osterhage, A. Wright, B. Schulz, S. Draeger, H.-J. Aust, R. Kaminsky, G. M. König; New Natural Products from Alga- and Sponge-associated fungi. Poster presented at 12. Irseer Naturstofftage der DECHEMA e.V., Germany, 23-25 February 2000.

C. Klemke, C. Osterhage, G. M. König, A.D. Wright; Natural products from fungi associated with marine organisms. Poster presented at DPhG Jahrestagung, Münster, Germany, 5-7 October 2000.

Zuccaro, G. M. König, A. D. Wright, C. Osterhage, H.-J. Aust, S. Draeger, C. Boyle, B. Schulz; Endophytic fungi of marine algae as a source of biologically

active secondary metabolites. Poster presented at 13. Irseer Naturstofftage der DECHEMA e.V., Germany, 28 February -2 March 2001.

Patente / Patents

Stadler, M., Hellwig, V., Gehling, M., Endermann, R., Bauch, F., Osterhage, C., König, G. M., Wright, A. D.; Ascosalipyrrolones A and B; DE 199 62 933.1, 24.12.1999.

Acknowledgements

I wish to express my gratitude to Prof. Dr. G. M. König and Dr. A. D. Wright for the sincere support they gave in all phases of my project.

I would like to thank Prof. Dr. H.-J. Aust and all members of his group for a very friendly and synergistic cooperation. Special thanks go to Dr. B. Schulz and Dr. S. Draeger for their special advice and help throughout.

Special thanks also go to Dr. U. Höller, University of Iowa at Iowa City, USA, for introducing me to the world of fungi, for explaining standard working procedures to me and for having amusing as well as serious discussions.

This study involved many different specific tasks, e.g., collection and taxonomic identification of the algae, recording of various spectra of isolated compounds, and biological testing, which were in part performed in cooperation with other research groups. For this work, thanks go to:

Dr. A. Flores-Moya, Depatamento de Biologia Vegetal, Facultad de Ciencias, Universidad de Málaga, Spain, and Dr. I. Bartsch, Biologische Anstalt Helgoland in der Stiftung Alfred-Wegener-Institut für Polar- und Meeresforschung, for taxonomic identification of the algal samples.
Prof. Dr. T. Hartmann, Institut für pharmazeutische Biologie, TU Braunschweig, for taxonomic identification of other plants.
PD Dr. U. Karsten, Alfred-Wegener-Institut für Polar- und Meeresforschung, Bremerhaven, for collecting the algal samples at Spitzbergen, Arctic.
T. Osterhage, U. and K. Zoller for organizing the collecting trip in summer 1997 in Moraira, Spain.
Dr. U. Höller for collecting algal samples from the Bretagne, France.

PD Dr. U. Engelhardt, Institut für Lebensmittelchemie, TU Braunschweig, for the use of HPLC-TSP-MS instrumentation, and Dr. C. Lakenbrink and Dr. A. Kiehne for assistance with HPLC-TSP-MS measurements.
Dr. M. Schwibbe, Deutsches Primatenzentrum GmbH, Göttingen, for performing statistical calculations.
Ms. P. Holba-Schulz and Ms. I. Rübesamen, NMR Abteilung der Chemischen Institute, TU Braunschweig, for recording ^{1}H NMR spectra;
Dr. V. Wray, Ms. B. Jaschok-Kentner and Ms. C. Kokoschke, GBF Braunschweig, for recording NMR spectra.
Dr. H.-M. Schiebel, Dr. U. Papke and Ms. D. Döring, Massenspektrometrie der Chemie, TU Braunschweig, and Mrs. A. Waßmann, GBF Braunschweig, for mass spectral measurements.
Dr. L. Witte, Institut für pharmazeutische Biologie, TU Braunschweig, for GC-MS measurements and for his help with the interpretation of some of these spectra.
Prof. Dr. P. G. Jones, Institut für Anorganische und Analytische Chemie, TU Braunschweig, for performing the single crystal X-ray analysis of 5-Hydroxyramulosin.

Ms. P. Reich and Mr. T. Kroker, Institut für Pharmazeutische Chemie, TU Braunschweig, for recording all IR spectra.

PD Dr. P. Leseberg, Chemie Bibliothek, and Mr. S. Wulle, UB, TU Braunschweig, for conducting CAS online searches.

Dr. G. Kirsch, Ms. C. Klemke, Ms. I. Rahaus and Mr. C. Dreikorn for performing ELISAs and many agar diffusion assays.
Mr. L. Peters for testing extracts for nematicidal and brine shrimp lethal activities.
Dr. R. Kaminsky, Swiss Tropical Institute, Basel, Switzerland, for the determination of antiplasmodial and cytotoxic activities against rat skeletal muscle myoblast cells and mouse peritoneal macrophages and for investigating effects of pure compounds against human sleeping sickness (*Trypanosoma brucei* subsp. *rhodesiense*) and Chagas disease (*Trypanosoma cruzi*).
Prof. Dr. S. Franzblau and his research group, U.S. Department of Health and Human Services, GWL Hansen's Disease Centre, Baton Rouge, Louisiana, USA, for testing extracts for inhibition of *Mycobacterium tuberculosis*.

Ms. I. Müller, Ms. S. Wahler, and Mr. M. Herold who worked as undergraduate students at the Institute, for technical assistance with isolation of fungal strains, extraction of fungal cultures, and biological testing of resulting extracts.

I wish to express my gratitude to all members of the Institut für Pharmazeutische Biologie, TU Braunschweig, past and present, for their friendship and help over the years I spent in the institute, especially Mr. O. Papendorf, Ms. I. Rahaus, Mr. M. Wessels and Dr. E. Goclik. Mr. R. Harms, Prof. Dr. U. Eilert, Dr. R. Lindigkeit and Dr. R. Müller for the common joy looking after the students of the "Teekurs"-Praktikum.

Finally, I would like to thank my parents, Doris and Rainer, for being totally supportive of me throughout all of my studies.

Financial support from the Bundesministerium für Bildung und Forschung (BMBF), and the Bayer AG, Leverkusen, is gratefully acknowledged.

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Abbreviations

°C	Degrees Celsius
1D	One Dimensional
2D	Two Dimensional
[α]	Specific rotatory power
δ	NMR chemical shift [ppm]
λ	wavelength [nm]
µL	10^{-6} litre
µm	10^{-6} metre
ν	wave number [cm^{-1}]
ASW	Artificial Sea Water
B. m.	*Bacillus megaterium*
BIO	Biomalt agar
br	broad
c	concentration [g/100 mL]
C. f.	*Chlorella fusca*
c.f.	confer
CH_2Cl_2	Dichloromethane (see DCM)
CI	Chemical Ionisation
cm	10^{-2} metre
COSY	COrrelated SpectroscopY
CT	Collection Trip
d	doublet
DAD	Diode Array Detector
DCM	Dichloromethane
DEPT	Distortionless Enhancement by Polarisation Transfer
dest.	distilled
E. c.	*Escherichia coli*
e.g.	example given
E. r.	*Eurotium repens*
EI	Electron Ionisation
ELISA	Enzyme Linked Immuno Sorbent Assay
EtOAc	Ethyl acetate
EtOH	Ethanol
F. o.	*Fusarium oxysporum*
FAB	Fast Atom Bombardment
g	gramme
GC	Gas Chromatography
GPY	Glucose Peptone Yeast extract agar
HIV-1	Human Immunodeficiency Virus 1
HMBC	Hetero nuclear Multiple Bond Correlation
HMQC	Hetero nuclear Multiple Quantum Coherence
HPLC	High Performance Liquid Chromatography
HR	High Resolution
Hz	Hertz
IC	Inhibition Concentration

IR	Infrared
J	Spin-spin coupling constant [Hz]
KM	Kartoffel-Möhren [potato carrot] agar
L	Litre
m	multiplet (in connection with NMR data)
M.m.	*Mycotypha microspora*
M. v.	*Microbotryum violaceum*
MeOH	Methanol
MHz	Megahertz
min	minute
mL	10^{-3} litre
mM	10^{-3} Mol
mp.	melting point
MS	(microbiology) Malt extract Soya meal agar
MS	Mass Spectrometry
nm	10^{-9} metre
NMR	Nuclear Magnetic Resonance
NOE	Nuclear Overhauser Effect
ppm	parts per million
q	quartet
RI	Refractive Index
RP	Reversed Phase
RT	Room Temperature
RT	Reverse Transcriptase (in connection with HIV-1)
s	singlet
SNA	Standard Nutrient Agar
sp.	species
t	triplet
TK	Tyrosine Kinase
TLC	Thin Layer Chromatography
UV	Ultra Violet
VLC	Vacuum-Liquid Chromatography

1 Introduction

1.1 Fungi as a source of biologically active metabolites

Due to their pharmaceutical potential secondary metabolites of fungi have been studied for more than 70 years. The search for new drugs from fungi started with the discovery of penicillin (Fleming, 1929), a potent antibiotic against Gram-positive bacteria, which was produced *by Penicillium notatum*. A further milestone in the history of fungal products for medicinal use was the discovery of the immunosuppressant cyclosporine which is produced, e.g., by *Tolypocladium inflatum* and *Cylindrocarpon lucidum* (Dreyfuss *et al.*, 1976). It was first discovered as an antifungal metabolite and later found to be immunosuppressive which made cyclosporine useful for the treatment following organ transplantation (Goodman Gilman *et al.*, 1985). The antifungal agent griseofulvin being isolated from *Penicillium griseofulvum* (Rehm, 1980) and the cholesterol biosynthesis inhibitor lovastatin isolated from *Aspergillus terreus* (Alberts *et al.*, 1980) are two further examples supporting today's great interest in new secondary metabolites from fungi.

So far more than 4000 fungal metabolites are described (Dreyfuss & Chapela, 1994) and 5000-7000 taxonomic species have been studied with respect to their chemistry (Hawksworth, 1991). In 1995 Hawksworth estimated the probable number of existing fungi to be 1.5 Mio. with only 71.000 being described so far. Apparently, the majority of fungi inhabiting the world has not yet been described. This implicates fungi to represent an enormous source for natural products with diverse chemical structures and activities. Of special interest are creative fungal strains. Creativity in this sense is defined as the ability to produce compounds of interest for human activities (Dreyfuss & Chapela, 1994).

Even if the natural function of secondary metabolites often is unknown, it is assumed that they play an important role in chemical defence and communication (Krohn, 1996). Many of them have been suggested to act as pheromones, antifeedants or repellents, and as regulators in the development

of organism (Sterner, 1995). Gloer (1995) suggested that the biosynthesis of secondary metabolites does not occur randomly but is correlated with ecological factors.

Most fungi studied to date have been isolated from soil and were proven to have a high creativity index, i.e. new and interesting secondary metabolites could be isolated. Genera such as *Aspergillus*, *Penicillium*, *Acremonium*, *Fusarium*, all typical soil isolates, are known for their ability to synthesise diverse chemical structures. Dreyfuss (1986), however, described a problem which is often encountered during microbiological screening of fungal isolates for their secondary metabolite content. Increasingly, known metabolites are rediscovered making screening programs less efficient. This may be caused partially by using always the same well established isolation methods for fungi. Thus, often the same fungal strains are reisolated when investigating an ecological niche. This could also result in the rediscovery of known compounds as the same taxa produce the same metabolites with high coincidence (Loeffler, 1984).

The assumption can be made that certain physical and biological situations in the natural environment favour the production of a diverse range of secondary metabolites (Dreyfuss & Chapella, 1994). This indicates that it might be more useful to investigate fungal isolates from other ecological niches than soil in order to take a more direct approach towards creative and novel fungal groups. Some relatively unexplored fungal groups derived from such ecosystems are, e.g., fresh-water fungi, marine fungi and endophytic fungi (Dreyfuss & Chapella, 1994).

1.2 Marine fungi

The present work deals with higher marine fungi. The secondary metabolism of marine fungi is of special interest due to the scarce information about it. Also the increasing reisolation of known compounds made screening programs dependent on soil derived fungi less efficient and forced scientists to look for alternatives, i.e. unexplored ecological niches.

Nearly all forms of life in the marine environment, e.g., algae, sponges, corals, tunicates, nudibranches, have been investigated for their natural products content (Faulkner, 2000a and b). Sessile plants and animals without any mechanical protection were investigated with special interest, as they could be assumed to have a repertoire of chemical defence systems. These poisons often exhibited potent pharmacological activities in assay systems used for the search of new pharmaceuticals, e.g., halichondrin B from the sponge *Halichondria okadai* (Hirata & Uemura, 1986), and halomon from the red alga *Portieria hornemannii* (Fuller *et al*., 1992), two prominent cytostatic compounds which are in preclinical development. Already in 1849 *Phaeosphaeria typharus*, the first described facultative marine fungus, was isolated from freshwater, and in 1869 the first obligate marine fungus *Halotthia posidoniae* was obtained from the rhizomes of sea grass. However, chemists focused mainly on marine macroorganisms and neglected marine microorganisms. But resources concerning collectable marine organisms are limited and are thus likely to be almost completely explored within the next 20 years (Fenical, 1997). Thus, marine microorganisms as a huge resource for potential drugs are likely to attract even more attention.

The advantages of the investigation of microorganisms as compared to macroorganisms are obvious: biotechnological fermentations are possible without ecological exploitation, compounds can be reisolated after recultivation in large amounts which is nearly impossible for marine macroorganisms. Additionally, microorganisms can be easier manipulated genetically and finally, the investigation of an as yet relatively little examined

group of organisms promises the discovery of unprecedented natural products (König & Wright, 1997).

Marine fungi do not represent a specific taxa, but are a group defined by their ecology and physiology. They can be divided into obligate and facultative marine fungi. Kohlmeyer (1974) defined obligate marine fungi as those “that grow and sporulate exclusively in a marine or estuarine (brackish water) habitat; facultative marine are fungi from freshwater or terrestrial areas also able to grow in the natural marine environment.” This implicates that always the ability to germinate in the natural marine environment has to be assessed before defining a marine-derived fungal isolate as obligate or facultative marine. The isolation of a fungal strain from a marine sample does not prove that this fungus is active in the marine environment. It is always possible to isolate a terrestrial fungus being a contaminant. Possibly such a fungal isolate was dormant in the form of spores or hyphal fragments until the surrounding conditions in the laboratory became favourable for germination and growth. Most fungi isolated from marine samples are not proven to be obligate or facultative marine. Thus, the more general expression marine-derived fungi is used.

1.2.1 Secondary metabolites from obligate marine and marine-derived fungi (excluding fungi from algae, see 1.2.2)

Until 1991 Kohlmeyer & Kohlmeyer have listed 321 taxa of higher (obligate) marine fungi, namely 255 Ascomycotina, 6 Basidiomycotina and 60 mitosporic fungi. It is surprising that less than 5 % have been investigated for their secondary metabolite chemistry. Most of the described fungi were isolated from driftwood and mangrove wood, but also from sea mud, sand and coastal marsh grass (Kohlmeyer & Kohlmeyer, 1991). Unfortunately, in some reports concerning the chemistry of obligate marine fungi there is nothing reported about the origin of the fungi or it is merely referred to a culture collection (Abraham *et al.*, 1994; Alam *et al.*, 1996).

Marine-derived fungi have been shown to be tremendous sources for new and biologically active secondary metabolites (Liberra & Lindequist, 1995; König & Wright, 1996; Pietra, 1997; Biabini & Laatsch 1998) which is reflected by the increasing number of published literature dealing with compounds from this group of fungi. A prominent source for marine-derived fungi are sponges. It has to be noticed that most fungal isolates from sponges belong to genera typical for terrestrial habitats. As sponges filter sea water this is not surprising. By this way terrestrial fungal spores or fragments of hyphae might get enriched in sponges. Additionally, samples of tunicates, fishes, a coral, a shell of crab, sea sediment and driftwood were investigated for the presence of fungi. Table 1 provides a complete listing (literature from 1972 to May 2000) of all investigations on metabolites of marine-derived fungi. Investigations concerning secondary metabolites of obligate marine fungi are separated from this listing and are reported in the following section. This shall show distinctively that the rough term "marine-derived“ declares nothing but the environment the fungus was obtained from in contrast to the terms “obligate marine“ and “facultative marine“ fungi which are clearly defined by Kohlmeyer (see 1.2). It takes large scientific efforts to characterize a fungus as belonging to this group.

Table 1. Metabolites reported from marine-derived fungi (excluding those from obligate marine and algicolous fungi).

Fungus isolated & investigated	Source of fungus	Metabolite(s) isolated	Literature citation
Cephalosporium sp.	sea water	Cephalosporins	Flynn (1972)
Aspergillus sp.	ocean floor mud	Gliotoxin	Okutani (1977)
Penicillium sp.	marine sediment	Epolactaene	Kakeya *et al.* (1995)
Preussia aurantiaca	mangrove isolate, from culture collection	Auranticins A and B	Poch & Gloer (1991)
Penicillium fellutanum	gastrointestine of the marine fish *Apogon endekataenia*	Fellutamides A and B	Shigemori *et al.* (1991)
Phoma sp.	shell of crab *Chionoecetes opilio*	Phomactins A, B, B_1, B_2, C, D, E, F, G	Sugano *et al.* (1991, 1994, 1995) Sato (1996)
Stachybotrys sp.	brackish water	Stachybotrins A and B	Xu *et al.* (1992)
Aspergillus fumigatus	salt water fish *Pseudolabrus japonicus*	Fumiquinazolines A-G	Numata *et al.* (1992) Takahashi *et al.* (1995b)
Trichoderma harzianum	sponge *Micale cecilia*	Trichoharzin	Kobayashi *et al.* (1993)
unidentified ("perhaps a mixture of two fungi")	sponge *Stylotella* sp.	Nectriapyrones A-B	Abrell *et al.* (1994)
unidentified	sponge *Jaspis* aff. *johnstoni*	Chloriolins A-C, coriolin B, dihydrocoriolin C	Cheng *et al.* (1994)
unidentified	sea sediment	Acetophthalidin	Cui *et al.* (1996)
Microascus longirostris	sponge, taxonomy not given	Cathestatins A-C	Yu *et al.* (1996a)
Aspergillus ?ochraceus	sponge *Jaspis* cf. *coriacea*	Chlorocarolides A-B, *R*-mellein, penicillic acid, hexylitaconic acid	Abrell *et al.* (1996a)

Table 1 continued.

Fungus isolated & investigated	Source of fungus	Metabolite(s) isolated	Literature citation
Microsphaeropsis olivacea	sponge *Agelas* sp.	Cerebroside	Keusgen *et al.* (1996)
		10-Methyl-(9*Z*)-octadecenoic acid, 1-*O*-(10-methyl-(9*Z*)-octadecenoyl)-glycerol	Yu *et al.* (1996b)
Exophiala pisciphila	sponge *Mycale adhaerens*	Exophilin A	Doshida *et al.* (1996)
unidentified (951014)	sponge *Spirastrella vagabunda*	Secocurvularin	Abrell *et al.* (1996b)
Penicillium sp.	sea sediment	Acetophthalidin	Cui *et al.* (1996)
Periconia byssoides	Sea hare *Aplysia kurodai*	Pericosines A and B, macrosphelides C, E-H	Numata *et al.* (1997a)
Pithomyces sp.	tunicate *Oxycorynia fascicularis*	Pitholides A-D	Wang *et al.* (1997)
Aspergillus niger	sponge *Hyrtios proteus*	Asperazine	Varoglu *et al.* (1997)
Gymnascella dankaliensis	sponge *Halichondria japonica*	Gymnastatins A-C	Numata *et al.* (1997b)
		Gymnastatins A-E	Amagata *et al.* (1998a)
		Gymnasterones A-B	Amagata *et al.* (1998b)
Trichoderma harzianum	sponge *Halichondria okadai*	Trichodenones A-C, harzialactones A-B, *R*-mevalonolactone	Amagata *et al.* (1998c)
Penicillium sp.	marine sediment	Anthranilamide Derivate NI15501A	Onuki *et al.* (1998)
Aspergillus sp.	saline lake sediment	Aspergillamides A and B	Toske *et al.* (1998)
Fusarium sp.	driftwood in mangrove habitat	Neomangicols A-C	Renner *et al.* (1998)
Paecilomyces cf. *javanica*	sponge *Jaspis* cf. *coriacea*	Deoxynortrichoharzin	Rahbæk *et al.* (1998)

Table 1 continued.

Fungus isolated & investigated	Source of fungus	Metabolite(s) isolated	Literature citation
unidentified	sponge *Haliclona* sp.	Hirsutanols A-C, *ent*-gloeosteretriol	Wang *et al.* (1998)
Trichoderma longibrachiatum	sponge *Haliclona* sp.	vertinoid polyketides, epoxysorbicillinol	Sperry *et al.* (1998)
Coniothyrium sp.	sponge *Ectyplasia perox*	(3S)-(3',5'-dihydroxyphenyl)-butan-2-one, 2-(1'(E)-propenyl)-octa-4(E),6(Z)-diene-1,2-diol, (3R)-6-methoxymellein, (3R)-6-methoxy-7-chlormellein, cryptosporiopsinol, phenylethanol, (p-hydroxyphenyl) ethanol, 2-(hydroxymethyl)-furan	Höller *et al.* (1999)
Microsphaeropsis sp.	sponge *Myxilla incrustans*	Microsphaeropsisin, (R)-mellein, (3R, 4S)-hydroxymellein, (3R, 4R)-hydroxymellein, 4,8-dihydroxy-3,4-dihydro-2H-naphtalen-1-one	Höller *et al.* (1999)
Emericella unguis	mollusc/medusa	Guisinol	Nielsen *et al.* (1999)
		Unguisins A and B	Malmstrøm (1999)
unidentified (Mitosporic fungus)	soft orange coral	Spiroxins A-E	McDonald *et al.* (1999)
Fusarium sp.	seagrass *Halodule wrightii*	Sansalvamide	Belofsky *et al.* (1999)
Massarina tunicata	submerged twig	Massarinolins A-C	Oh *et al.* (1999)
Dendrospora tenella	freshwater	Tenellic acids A-D	Oh *et al.* (1999)
unidentified (I96S215)	orange sponge	*iso*-cladospolide B, *seco*-patulolide C, pandangolides 1 and 2, cladospolide B	Smith *et al.* (2000)

Table 1 continued.

Fungus isolated & investigated	Source of fungus	Metabolite(s) isolated	Literature citation
Penicillium steckii	unidentified tunicate	Tanzawic acids E and F, 3,7-dimethyl-1,8-dihydroxy-6-methoxyisochroman, 3,7-dimethyl-8-hydroxy-6-methoxyisochroman	Malmstrøm *et al.* (2000)
Aspergillus niger	sponge *Hyrtios proteus*	Asperic acid, hexylitaconic acid, malformin C, pyrophen, asperazine	Varoglu & Crews (2000)
Penicillium sp.	Marine bivalve *Mytilus coruscus*	Sculezonones A and B	Komatsu *et al.* (2000)
Drechslera hawaiiensis	sponge *Callyspongia aerizusa*	Spiciferones A and B, Spiciferol A	Edrada *et al.* (2000)
Penicillium sp.	Marine bivalve *Mytilus coruscus*	Coruscol A	Kagata *et al.* (2000)

The first report on the chemistry of obligate marine fungi in 1981 focused on *Halocyphina villosa*, a Basidiomycete living as a saprobe on the surface of wood (Kohlmeyer & Kohlmeyer, 1979). It was found to produce the antibiotic compound siccayne (Kupka *et al.*, 1981) which was already known from the terrestrial mitosporic fungus *Helminthosporum sativum* (Ishibashi *et al.*, 1968). The Ascomycete *Corollospora pulchella* was isolated from driftwood. Investigation for its secondary metabolite content (Furuya *et al.*, 1985) yielded the antibiotic compounds melinacidins III and IV, already known from *Acrostalagmus cinnabarinus* (Argoudelis & Reusser, 1971), the antitumour substance gancidin W also being produced by *Streptomyces gancidicus* (Aiso *et al.*, 1956), and pulchellalactame, a CD45 protein tyrosine phosphatase inhibitor (Alvi *et al.*, 1998). A culture of the lignicolous Ascomycete *Leptosphaeria oraemaris* was found to contain leptosphaerin (Schiehser *et al.*, 1986; Pollenberg & White, 1986; Rollin, 1987; White *et al.*, 1989), obioninene and oraemarin (Miller & Savard, 1989), leptosphaerolide and leptosphaerodione (Guerriero *et al.*, 1991). *Zopfiella marina*, which was

isolated from sea mud, was found to produce zopfinol (Kondo *et al.*, 1987), and a culture of the Mitosporic fungus *Asteromyces cruciatus*, occuring on wood and decaying marine algae, was found to contain gliovictin (Shin & Fenical, 1987). From the saprobic *Dendryphiella salina* dendryphiellin A, A1, B, C, D, E, E1, E2, F, and G and dendryphiellic acids A and B and glyceryl dendryphiellate A could be obtained (Guerriero *et al.*, 1988, 1989, 1990). *Heliascus kanaloanus*, a mangrove Ascomycete, was reported to produce heliascolides A and B (Poch & Gloer,1989a) and the known ochracin (Cole & Cox, 1981). *Leptosphaeria obiones*, which was isolated from coastal marsh grass, was found to produce obionin A (Poch & Gloer, 1989b). A cytotoxic compound together with 7-hydroxyergosterol and a ceramide could be purified from the extract of *Lignincola laevis* (Abraham *et al.*, 1994). Takahashi *et al.* (1994a and b, 1995a) reported the isolation of leptosins A, B, C, D, E, F, G, G_1, G_2, H and I from a *Leptosphaeria* sp., an epiphyte on the marine alga *Sargassum tortile* (see also 1.2.2). Leptosins A and C exhibited significant antitumour activity against sarcoma 180 ascites. Leptosins G, G_1, G_2, H, I and J showed cytotoxic activity towards cultured P 388 cells. *Cirrenalia pygmea* was found to contain melanin as protection against changes in osmotic pressure (Ravishankar *et al.*, 1995). From the marine Ascomycete *Kallichroma tethys* isoculmorin was isolated (Alam *et al.*, 1996). In 1998 the obligate marine Ascomycete *Corollospora maritima*, which occurs on rotting algae or driftwood, was reported to produce corollosporine (Liberra *et al.*, 1998) and a strain of *Hypoxylon oceanicum* isolated from mangrove wood was found to produce lipodepsipeptides and macrocyclic polylactones with lipodepsipeptide 15G256γ being an antifungal antibiotic, inhibiting cell wall biosynthesis (Schlingmann *et al.*, 1998).

Even if the structures of some natural compounds isolated from marine-derived fungi were new, the hope for completely different structural types was not met. Too often secondary metabolites isolated were already known from terrestrial organisms. A more successful approach for finding new compounds could be to isolate fungal strains from marine ecological niches where fungi

are forced through selection pressures to produce specialised secondary metabolites, helping them to survive.

1.2.2 Secondary metabolites from algicolous fungi

Almost one-third of all known higher marine fungi are associated with algae. Fungi are usually restricted to one class of algae, namely, Chlorophyta, Phaeophyta or Rhodophyta. Most algicolous fungi are Ascomycetes, only few Mitosporic fungi are known (Jones, 1976; Kohlmeyer & Kohlmeyer, 1979; Stanley, 1992). Although the first descriptions of algae-inhabiting fungi appeared at the end of the nineteenth century most mycologists ignored this topic and concentrated on wood-inhabiting fungi. The preference for lignicolous fungi over algicolous ones as research objects can be explained by the easier accessibility of wood. Additionally, algae-inhabiting fungi occur in a relatively sporadic way which can be explained by antibiotic substances, e.g., tannins being produced by healthy algae. Finally, enormous difficulties are encountered when growing host algae in laboratory cultures for infection experiments. These investigations are necessary in order to interpret the host-plant relationship as algicolous fungi can be symbionts, parasites or saprobes.

The main focus of the present study lies on the investigation of the secondary metabolism of endophytic fungi from algae. The reason for the investigation of fungal endophytes for their secondary metabolite content is clear, if considering that one way in which a fungus can play a mutualistic role is to excrete metabolites which are toxic for pathogens of its host (Schulz *et al.*, 1995). These are often biologically active compounds, e.g., endophytes derived from fir needles produced toxic metabolites to spruce bud-worm (Calhoun *et al.*, 1992), and alkaloids conferring resistance to herbivory (Leuchtmann, 1992).

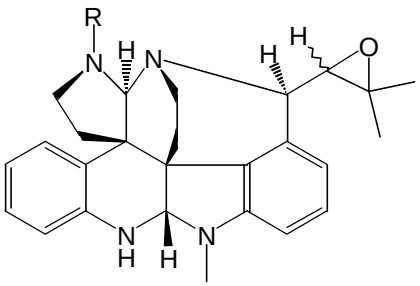
Fungal isolates from algae often belong to representatives classically known for the terrestrial habitat as revealed by *Penicillium* spp., *Aspergillus* spp., *Fusarium* spp. and *Acremonium* species. Though most of these fungal strains

are not obligate marine fungi, nearly all compounds isolated from them in the last five years have novel chemistry.

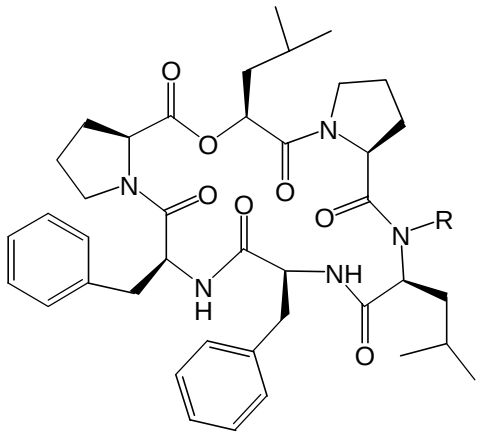
A complete listing of the literature from 1993 to May 2000 of metabolites found in algicolous fungi is provided in Table 2. Table 2 also demonstrates the taxonomy of fungi that can be found in algae. Chemical structures of compounds are given in Figure 1 (in alphabetical order).

Table 2. Metabolites reported from algae-derived fungi.

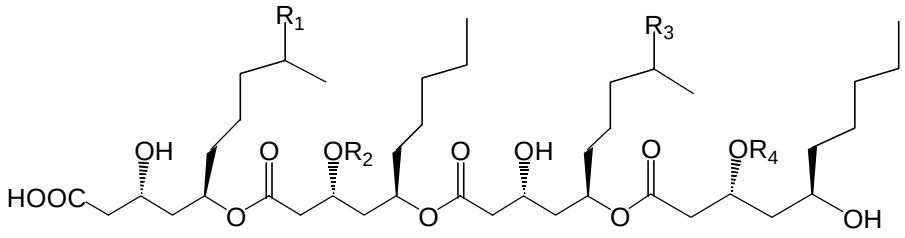
Algal species investigated	Fungus isolated & investigated	Metabolite(s) isolated	Literature citation
Enteromorpha intestinalis	*Penicillium* sp.	Communesins A and B	Numata *et al.* (1993)
Sargassum tortile	*Leptosphaeria* sp.	Leptosins A-F	Takahashi *et al.* (1994a)
		Leptosins G, G_1, G_2 and H	Takahashi *et al.* (1995a)
		Leptosins I and J	Takahashi *et al.* (1994b)
Enteromorpha intestinalis	*Penicillium* sp.	Penochalasins A-C	Numata *et al.* (1996)
		Penostatins A-D	Takahashi *et al.* (1996)
		Penostatins F-I	Iwamoto *et al.* (1998)
		Penostatins A-E	Iwamoto *et al.* (1999)
Halymenia dilatata	*Fusarium* sp.	Halymecins A-C	Chen *et al.* (1996)
	Acremonium sp.	Halymecins D, E	Chen *et al.* (1996)
unidentified green alga, decaying leaf of *Rhizophora manglei*, *Penicillus* sp., *Batophora* sp.	*Aspergillus insulicola* (five strains)	Insulicolide A, asteltoxin	Rahbæk *et al.* (1997)
Ceramium condi	*Cladosporium sphaerospermum*	two sterols	Kuznetsova *et al.* (1998)
Penicillus capitatus	*Aspergillus versicolor*	4 sesquiterpenoid nitrobenzoyl esters	Belofsky *et al.* (1998)
Sargassum ringgoldianum	*Penicillium waksmanii*	Pyrenocines D, E, pyrenocines A, B, three dioxopiperazine derivatives	Amagata *et al.* (1998d)
decaying plant material	*Scytalidium* sp.	Exumolides A, B	Jenkins *et al.* (1998a)
Halimeda monile	unidentified	Solanapyrones C, E-G	Jenkins *et al.* (1998b)
Sargassum sp.	*Aspergillus* sp.	Mactanamide	Lorenz *et al.* (1998)
Ceratiodictyon sporgiosum	unidentified	Seragakinone A	Shigemori *et al.* (1999)



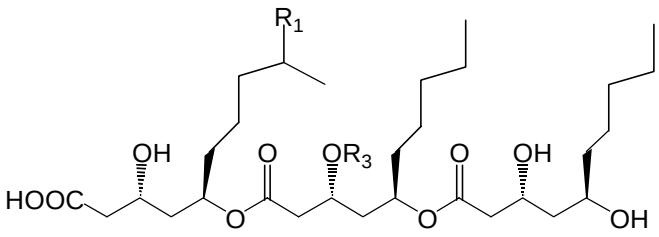
Communesin A R = $COCH_3$
Communesin B R = $COCH{=}CHCH{=}CHCH_3$
trans trans



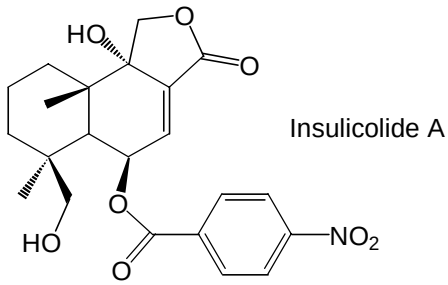
Exumolide A R = CH_3
Exumolide B R = H



Halymecin A R_1 = H, R_2 = Ac, R_3 = H, R_4 = H
Halymecin B R_1 = H, R_2 = Ac, R_3 = H, R_4 = β-D-mannosyl
Halymecin D R_1= OH, R_2= H, R_3 = OH, R_4 = H

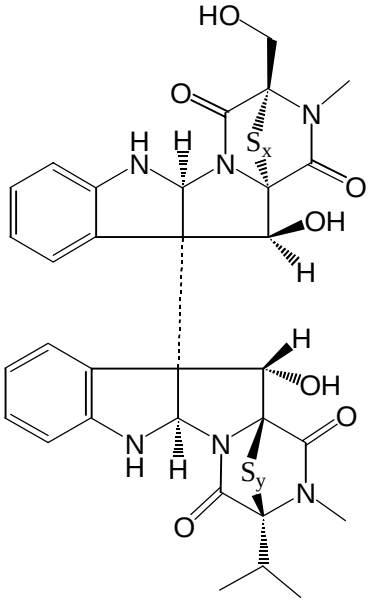


Halymecin C R_1 = H, R_2 = Ac
Halymecin E R_1 = OH, R_2 = H



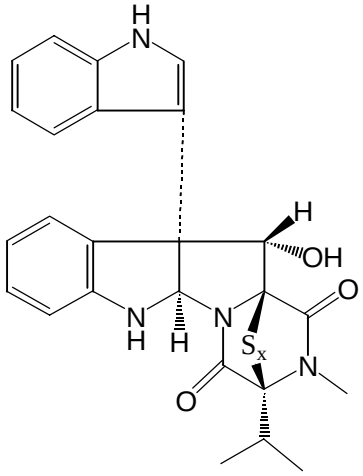
Insulicolide A

Figure 1: Metabolites from algae-derived fungi.

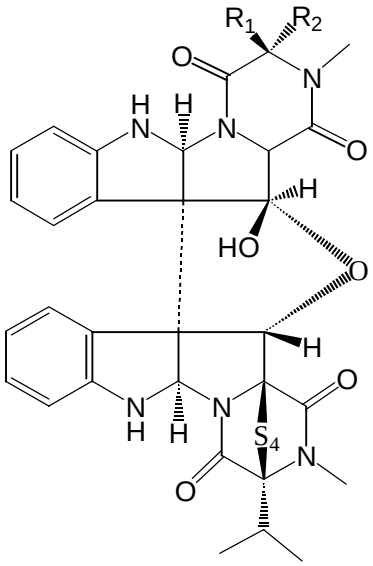


Leptosin A x = 4, y = 2
Leptosin B x = 3, y = 2
Leptosin C x = 2, y = 2

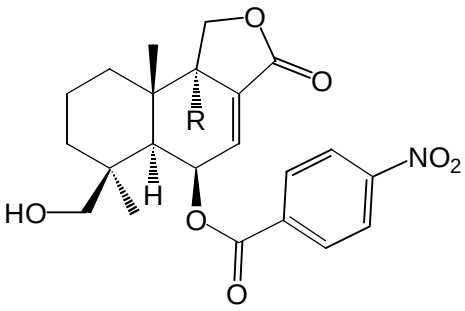
Leptosin G x = 4, y = 3
Leptosin G_1 x = 3, y = 3
Leptosin G_2 x = 2, y = 3
Leptosin H x = 2, y = 4



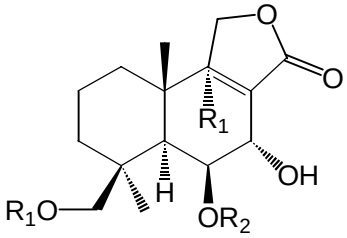
Leptosin D x = 2
Leptosin E x = 3
Leptosin F x = 4



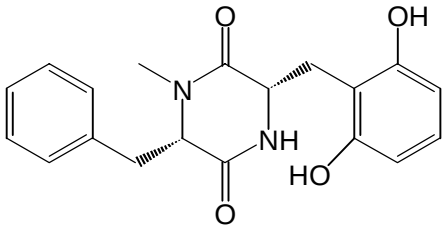
Leptosin I $R_1 = CH_2OH$, $R_2 = H$
Leptosin J $R_1 = H$, $R_2 = CH_2OH$



sesquiterpenoid nitrobenzoyl esters
R = OH
R = H

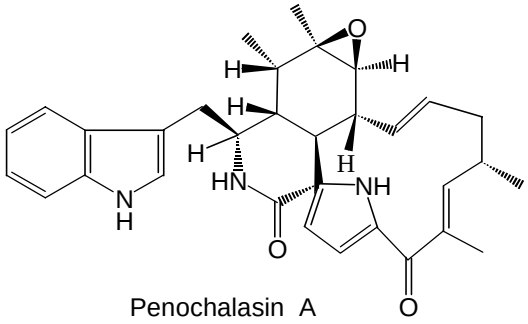


sesquiterpenoid nitrobenzoyl esters
R_1 = H, R_2 = *p*-nitrobenzoyl
R_1 = *p*-nitrobenzoyl, R_2 = H

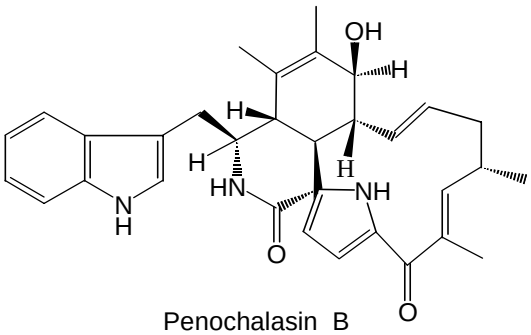


Mactanamide

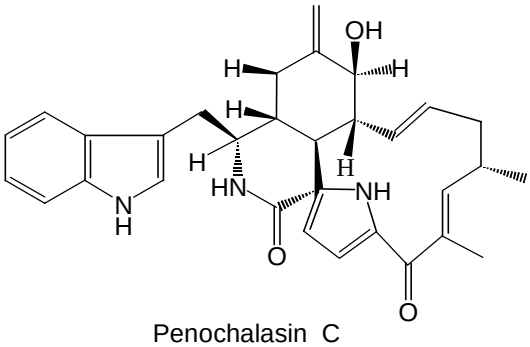
Figure 1 (continued): Metabolites from algae-derived fungi.



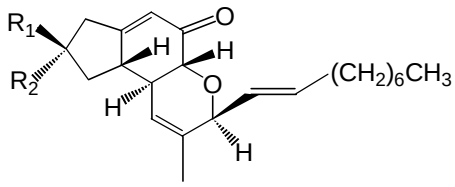
Penochalasin A



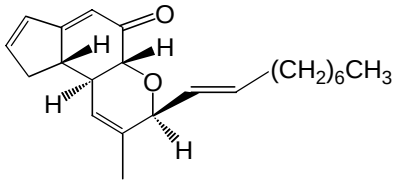
Penochalasin B



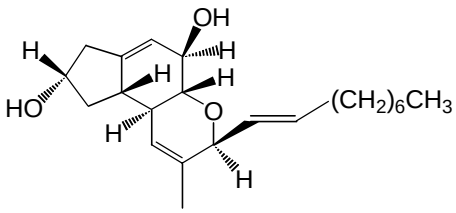
Penochalasin C



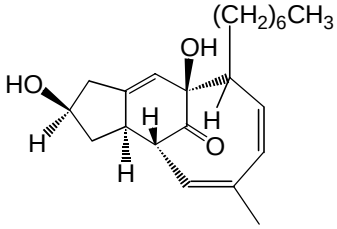
R_1 = OH, R_2 = H Penostatin A
R_1 = H, R_2 = OH Penostatin B



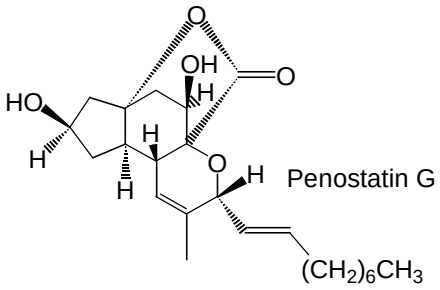
Penostatin C



Penostatin D

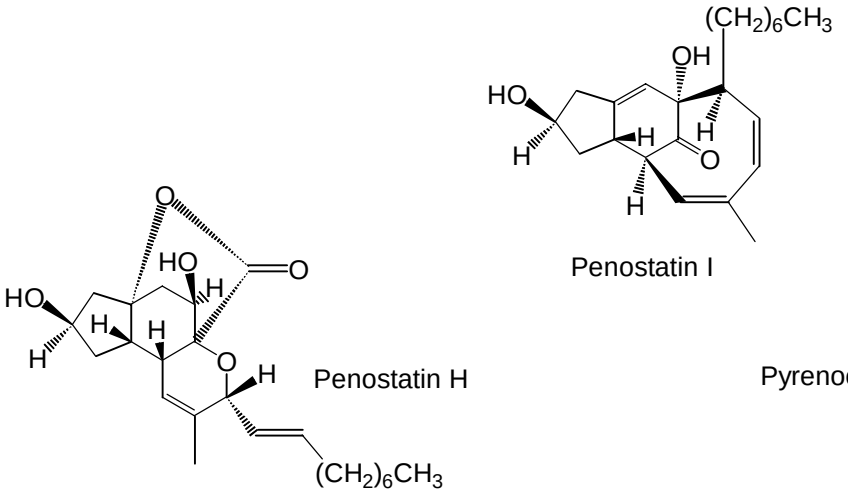


Penostatin F



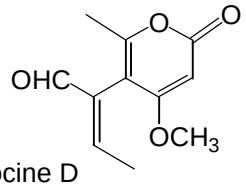
Penostatin G

Figure 1 (continued): Metabolites from algae-derived fungi.

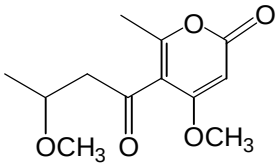


Penostatin H

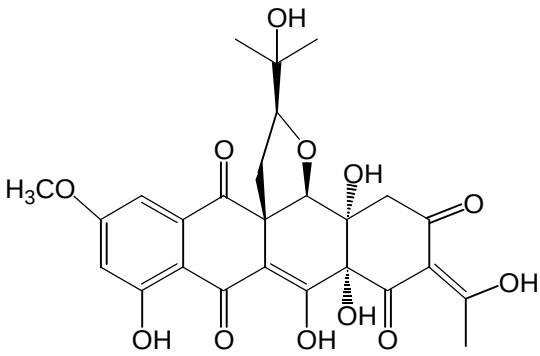
Penostatin I



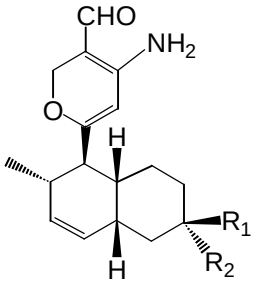
Pyrenocine D



Pyrenocine E



Seragakinone A



Solanapyrone E R_1 = OH R_2 = H
Solanapyrone F R_1 = H R_2 = OH
Solanapyrone G R_1 = H R_2 = H

Figure 1 (continued): Metabolites from algae-derived fungi.

2 Scope of the present study

The focus of the present study was the isolation of marine-derived fungi, especially from algae, and the investigation of their secondary metabolism. The project aimed at finding fungal strains being able to produce structurally novel and biologically active secondary metabolites for pharmaceutical purposes.

2.1 Differences between marine and terrestrial fungi of the genus *Phoma*

This part of the work served the purpose to find out whether marine-derived *Phoma* spp. differ significantly from terrestrial *Phoma* spp. with respect to their secondary metabolite content. Chromatographic analysis of fungal extracts in combination with diode-array detection (DAD) and thermospray mass spectrometry (TSP-MS) was performed. Retention times, UV- and mass spectral data in combination with statistical methods were used to investigate distinctions in metabolism of these two fungal groups.

2.2 Isolation of fungi from marine sources

This part of the work dealt with the isolation of fungal strains from marine organisms, mainly algae. Isolation methods were modified in order to isolate fungi adapted to the marine milieu. This aimed at finding fungi which have not yet been investigated for their secondary metabolite content.

2.3 Biological and chemical screening of fungal extracts

Fungal strains were to be taxonomically identified. Isolates belonging to typically marine genera, or such belonging to terrestrial genera known to produce interesting secondary metabolites were selected for further investigations. Small scale cultivation and extraction enabled biological activity testing. These tests mainly consisted of agar diffusion assays for antibacterial, antifungal and antialgal activity, and ELISA based test systems for inhibition of tyrosine kinase $p56^{lck}$ and HIV-1 reverse transcriptase. An additional investigation by TLC was used to further characterize the fungal culture extracts concerning number and amount of secondary

metabolites. The results obtained with these test systems were used for the selection of strains for detailed chemical investigations.

2.4 Chemical investigations of fungal strains

Special attention was paid to fungal isolates exhibiting prominent biological activity in one of our bio-assays. Large-scale cultivation, extraction, and separation using diverse chromatographic methods, mainly HPLC, was to be employed, in order to isolate pure metabolites. Bio-assay guided isolation was to be attempted, in order to identify the active compounds. Structure elucidation was performed using mainly 1D and 2D NMR techniques and by physical characterisation of the isolated metabolites.

2.5 Pharmacological potential of isolated pure compounds

Each pure compound obtained during this study was to be tested in as many different bio-assays as possible. Especially antimicrobial, antiplasmodial and enzyme inhibitory activities were to be evaluated with the help of collaborating scientists.

3 Materials and methods (general procedures)

Most materials and methods used during this study are described in the "Description of partial projects" chapter as they are of special interest for the respective study. Here general materials and methods are described which were used in many or all partial projects in order to avoid repetitions.

3.1 Chromatography

3.1.1 Thin layer chromatography (TLC)

TLC was carried out using either TLC aluminium sheets silica gel 60 F_{254} (Merck 5554) or pre-coated TLC plates SIL RP-18W / UV 254 (Macherey-Nagel). Standard chromatograms of fungal extracts were prepared by applying 20 µL solution (5 mg/mL) to a silica gel TLC plate and developing it with DCM/MeOH (95/5; v/v) under saturated conditions. Chromatograms were detected under UV light (254 and 366 nm), and with vanillin-H_2SO_4 reagent (1 g vanillin dissolved in 100 ml H_2SO_4, heated at 100 °C after spraying).

3.1.2 Vacuum-liquid chromatography (VLC)

Sorbents for VLC were either silica gel 60 (0.063-0.200 mm, Merck 7734; normal-phase) or Polyprep 60-50 C18 (Machery-Nagel 71150; reversed-phase). Columns were filled with the appropriate sorbent soaked with petroleum ether or MeOH. Before applying the sample, the column was equilibrated with the first designated eluent.

3.1.3 High performance liquid chromatography (HPLC) and detectors

HPLC was carried out either using a Merck-Hitachi system equipped with a L-6200A Intelligent Pump, a L-4500A diode array detector, a D-6000A interface with D-7000 HSM software, a Rheodyne 7725i injection system, or a system equipped with a Waters associates chromatography pump, a Knauer Differential refractometer, a Rheodyne 7725i injection system and a Linseis L 200 E recorder. If not stated otherwise, the system with differential refractometer as detector was used. Columns

used were either a Knauer Spherisorb S ODS 2 (5 µm, 250 × 8 mm), a Merck LiChrospher Si 60 (5 µm, 250 × 7 mm), or similar columns. Typical flow rates were 2.0 - 3.0 mL/min. All solvents, except H_2O, were distilled prior to use. The eluents were degassed under reduced pressure. If not stated otherwise, samples were diluted in the eluent to yield solutions of 20 - 40 mg/mL. Injected amounts usually equalled 10 mg of extract/run.

3.2 Structure elucidation

Structures were elucidated mainly using one and two dimensional NMR techniques and various MS methods. If necessary, additional parameters such as optical rotation, UV and IR properties were determined. Identity of isolated compounds with compounds reported in literature was judged, if not stated otherwise, based on ^{1}H and ^{13}C NMR spectroscopic data, and specific optical rotation. For some known compounds, mainly those first reported before 1980, no or no complete NMR data have been published. Furthermore, in some cases the published values were found to be insufficient to prove the identity of an isolated metabolite with the published compound in question. In these cases, a complete structure elucidation was performed. Literature searches were done using Chapman & Hall Natural Products on CD-ROM (Buckingham, 1996, 1998) and Beilstein on-line databases. Structures were designated as new, if they could not be found in Beilstein database and Chemical Abstracts.

3.2.1 NMR spectroscopy

^{1}H NMR spectra of extracts and pure compounds as a purity check were recorded at the Department of Chemistry, TU Braunschweig, on a Bruker AC-200 spectrometer operating at 200 MHz or a Bruker AM-400 spectrometer operating at 400 MHz (with CD_3OD as solvent) by Ms. P. Holba-Schulz and Ms. I. Rübesamen. ^{1}H NMR spectra of pure compounds and all other NMR measurements were performed at the GBF Braunschweig by Dr. V. Wray, Ms. B. Jaschok-Kentner and Ms. C. Kokoschke on Bruker DPX-300, ARX-400 or DMX-600 NMR spectrometer operating at 300, 400 or 600 MHz (^{1}H), and 75.5, 100 or 150 MHz (^{13}C), respectively. Spectra of pure compounds were processed using Bruker 1D WIN-NMR or 2D WIN-NMR software.

They were calibrated using solvent signals (^{13}C: $CDCl_3$ 77.00 ppm, CD_3OD 49.00 ppm, $\underline{C}D_3CO\underline{C}D_3$ 30.50 ppm) or a signal of the portion of the partly or not deuterated solvent (^1H: $CHCl_3$ in $CDCl_3$ δ 7.26 ppm, CH_3OD in CD_3OD δ 3.35 ppm, acetone in acetone-d_6 δ 2.05 ppm). Multiplicity for ^{13}C was deduced from DEPT experiments; s = C, d = CH, t = CH_2, q = CH_3. Structural assignments were based on spectra resulting from one or more of the following NMR experiments: ^1H, ^{13}C, DEPT, ^1H-^1H COSY, ^1H-^{13}C direct correlation (HMQC), ^1H-^{13}C long-range correlation (HMBC), and ^1H difference NOE.

3.2.2 Mass spectrometry

Mass spectral measurements were performed by Dr. H.-M. Schiebel, Dr. U. Papke and Ms. D. Döring (all Department of Chemistry, TU Braunschweig) using a Finnigan MAT 8430 spectrometer (EI, 70 eV; CI; FAB, Xe).

GC-MS analyses were performed by Dr. L. Witte using a Carlo-Erba HRGC 5160 linked to a Finnigan MAT 4515 spectrometer (40 eV). The GC was equipped with a DB1-30W column (30 m × 0.32 mm i.D., stationary phase 0.25 μm; split 1:20). Oven temperature program was modified for each individual sample but usually started at 100 °C, increasing 6 °C/min.

3.2.3 UV measurements

UV spectra were recorded on a Shimadzu UV-200S double beam spectrophotometer coupled with a Kipp & Zonen BD-40 recorder using 1.000 cm quartz cells.

3.2.4 IR spectroscopy

IR spectra were recorded by Ms. P. Reich and Mr. T. Kroker, Institute for Pharmaceutical Chemistry, TU Braunschweig, on an ATI Mattson Genesis Series FTIR. Samples were measured on NaCl (film).

3.2.5 Optical rotation

Optical rotations were measured using a Perkin Elmer 241 Polarimeter equipped with an 1 mL cell, cell length 10.000 cm.

3.2.6 Melting point

Melting points were recorded on a Wena thermoblock apparatus heated by a small Bunsen burner and are not corrected.

3.2.7 X-ray analysis

The single crystal X-ray analysis of 5-Hydroxyramulosin (**22**) was performed by Prof. Dr. P. G. Jones, Institute for Inorganic and Analytical Chemistry, TU Braunschweig. Crystals were grown from MeOH.

3.3 Origin and taxonomy of algal samples

Marine algal samples investigated in this study originated from eleven different locations, including temperate (North Sea, the Baltic Sea, Germany and Bretagne, France), subtropical (Azores and Costa Blanca, Spain) and arctic (Spitzbergen, Norway) regions. All samples were collected in the intertidal zones or by snorkelling. Voucher specimens are located at the Institut für pharmazeutische Biologie, University of Bonn, under the respective number.

All algal samples were stored in sterile sea water supplied with 250 mg/L benzylpenicillin and 250 mg/L streptomycin sulphate at 4 °C for a few days to one week until examination.

a) Samples from Germany:

Fucus vesiculosus (N-1); *Enteromorpha* sp. (N-3); *Ulva* sp. (N-4), Tönning, North Sea.

Laminaria sp. (CUX-1); *Porphyra* sp. (CUX-2); *Fucus vesiculosus* (CUX-3), Cuxhaven, North Sea.

Fucus spiralis (WI-1); *Fucus vesiculosus* (WI-2); *Porphyra* sp. (WI-3); *Enteromorpha* sp. (WI-4), Wilhelmshaven, North Sea.

Delesseria sanguinea sp. (HE-1); unidentified brown alga, (HE-2); *Petalonia fascia* (HE-3), Helgoland, North Sea.

Cladophora sp. (SY-1); *Enteromorpha* sp. (SY-2); *Enteromorpha* sp. (SY-3); unidentified red alga (SY-4), Sylt, North Sea.

Petalonia zosterifolia (OS-1); *Enteromorpha* sp. (OS-2); *Fucus vesiculosus* (OS-3); *Enteromorpha* sp. (OS-4), Fehmarn, the Baltic Sea.

b) Samples from Spain:

Cladophora pellucida (S-3); *Enteromorpha linza* (S-4); *Cladostephus spongiosus* (Hudson) C. Agardh (M-1); *Laurencia obtusa* (Hudson) Lamouroux (M-2); *Cystoseira* sp. (M-3); *Acetabularia acetabulum* (Linnaeus) Silva (M-4); *Liagora viscida* (Forskkal) C. Agardh (M-5); *Herposiphonia* sp. (M-6); *Jania rubens* (Linnaeus) Lamouroux (M-7), Moraira, Mediterranean Sea.

c) Samples from Portugal:

Osmundia pinnatifida (AZ-1); *Asparagopsis taxaformis* (AZ-2); *Valomia utricularis* (AZ-3); *Polysiphonia* cf. *fucoides* (AZ-4); *Cystoseira foeniculaceus* (AZ-5); *Dictyota* sp. (AZ-6); *Pterocladia capillacea* (AZ-8); *Corallina elongata* (AZ-9); *Enteromorpha* sp. (AZ-10); *Sargassum* sp. (AZ-11); *Cystoseira foeniculaceus* (AZ-12); *Cystoseira abeis-marina* (AZ-13); *Fucus spiralis* (AZ-14), Azores, Atlantic Ocean.

d) Samples from France (Bretagne, Atlantic Ocean):

*Sargas*sum *runticum* (BR-1); *Polyides rotundus* (BR-2); *Laminaria* sp. (BR-3); *Mastocarpus stellatus* (Stackhouse) Guiry (BR-4).

e) Samples from Norway (Spitzbergen, Arctic Ocean):

Laminaria digitata (AWI-1); *Laminaria sacchorhiza* (AWI-2); *Desmarestia anceps* (AWI-3); *Desmarestia menziesii* (AWI-4); *Adenocystis utricularis* (AWI-5); *Gigartina skottsbergii* (AWI-6); *Iridaea cordata* (AWI-7); *Ascoseira mirabilis* (AWI-8).

Origin and taxonomy of the sponge sample

Tedania anhelaus Lieberkuhn, 1859 (Poecilosclerida, Tedaniidae) (AZ-7), Azores, Atlantic Ocean, Portugal.

Origin and taxonomy of the jellyfish sample

Aurelia aurita (N-7), Tönning, North Sea, Germany.

Origin and taxonomy of plant samples

Salicornia europaea, Chenopodiaceae (N-2); *Limonium vulgare*, Plumbaginaceae (N-5); *Amophila arenaria*, Poaceae (N-6), Tönning, North Sea, Germany.

Posidonia oceanica (Linnaeus) Delile, Spermatophyta (S-1 and S-2); roots of *Posidonia oceanica* (Linnaeus) Delile, Spermatophyta (S-5), Moraira, Mediterranean Sea, Spain.

Preparation of animal and plant material

Algal samples were placed in EtOH (70 %) or first in EtOH (70 %) and then additionally in sodium hypochlorite solution (2 %), rinsed three times with sterile ASW to remove the EtOH, cut aseptically into small pieces and placed on isolation media. Surface sterilization time was optimised for each alga before placing cubes onto isolation medium, using approximately a third of the algal material for a surface sterilization test sequence.

Small pieces of inner tissue of sponge and jellyfish material were rinsed three times with sterile artificial sea water (ASW) and then aseptically cut into small cubes,

approx. $(0.5\ cm)^3$. A total of 15 - 20 cubes of each sample was placed on isolation media.

3.4 Media and inoculation conditions

Medium I: blended alga (the same sp. as used for isolation of fungi) 20 g/L, agar 15 g/L, sea water from the collecting site of the alga 1000 mL/L. This medium was supplemented with 250 mg/L benzylpenicillin and 250 mg/L streptomycin sulphate to prevent bacterial growth.

Medium II: Standard nutrient agar (SNA): KH_2PO_4 1 g/L, KNO_3 1 g/L, $MgSO_4 \times 7\ H_2O$ 0.5 g/L, KCl 0.5 g/L, glucose $\times\ H_2O$ 0.2 g/L, sucrose 0.2 g/L, agar 20 g/L.

Medium III: Glucose peptone yeast extract agar (GPY): glucose $\times\ H_2O$ 1 g/L, peptone from soymeal 0.5 g/L, yeast extract 0.1 g/L, agar 15 g/L, ASW 800 mL/L.

Medium IV: Potato carrot agar (KM): cooked and mashed potatoes 20 g/L, cooked and mashed carrots 20 g/L, agar 20 g/L, ASW 800 mL/L.

Medium V: Cornmeal agar: 42 g cornmeal was stirred in 500 mL distilled water at 60 °C for 12 h, filtered and the filtrate diluted with water to 1 L. To this solution 15 g agar and the salts contained in 800 mL ASW were added.

Artificial sea water (100 % ASW) contained the following salts (g/L): KBr 0.1, NaCl 23.48, $MgCl_2 \times 6\ H_2O$ 10.61, $CaCl_2 \times 2\ H_2O$ 1.47, KCl 0.66, $SrCl_2 \times 6\ H_2O$ 0.04, Na_2SO_4 3.92, $NaHCO_3$ 0.19, H_3BO_3 0.03. ASW 80 % means that of these salts only 80 % per litre are contained in water.

For the inoculation of algal samples medium I was used if the amount of alga needed was available; otherwise algal samples were placed onto agar plates containing only sea water, agar (15 g/L) and antibiotics (250 mg/L benzylpenicillin and 250 mg/L streptomycin sulphate).

Samples were incubated at RT (approx. 20 °C) except for those from Spitzbergen, which were additionally incubated at 4 °C, and regularly examined under a dissection microscope for the presence of developing fungal hyphae. Fungal colonies were transferred to one or more of the media for identification. These included media II, III, IV and V.

Identification of fungal strains

Isolated fungal strains were identified by Dr. S. Draeger, Institute for Microbiology, TU Braunschweig. Because of the great number of isolates, in most cases only the genus of the strains was determined. A species determination was only attempted for strains further investigated for their secondary metabolite chemistry.

Preservation and maintenance of stock cultures

All fungal strains were kept on two different media in test-tubes in duplicate at 4 °C:

Medium 1 (One tube per strain, 7 mL medium, sealed with cotton wool): Biomalt 50 g/L, agar 20 g/L, dest. H_2O 1 L.

Medium 2 (One tube per strain, 7 mL medium, sealed with aluminium cap and parafilm): glucose × H_2O 1 g/L, peptone from Soya meal 0.5 g/L, yeast extract 0.1 g/L, agar 15 g/L, ASW 800 mL/L (GPY).

Strains for further chemical investigations were kept in additional five tubes with medium 1 (medium 2 for obligate marine species), sealed with aluminium-cap and parafilm. Slants were prepared and inoculated using standard microbiological techniques.

Strains for screening purposes

Isolates were inoculated on the three following media (50 mL each) for 28 days at RT (20 °C):

Medium A (B): biomalt 20 g/L, agar 6.8 g/L.

Medium B (MS): Malt extract Soya meal agar (MS): malt extract 30 g/L, peptone from Soya meal 3 g/L, agar 7.6 g/L, ASW 800 mL/L.

Medium C (Bfl): biomalt 20 g/L, ASW 800 mL/L.

Liquid cultures were shaken on an Infors Novotron rotary shaker at 65 rpm. After four weeks, cultures were homogenised using an Ika Ultra-Turrax T 25 at 8000 rpm for 2 min. 50 mL of water were added to solid cultures prior to homogenisation. Resultant mixtures were extracted with EtOAc (3 × 50 mL), the organic fractions were combined, and the solvent removed at reduced pressure at 30 °C. Residues were redissolved in a mixture of acetone/MeOH (1:1; v/v) to a concentration of 5 mg/mL and this solution was then used for the agar diffusion assays and TLC. For the ELISA based assays 500 μg extract was dissolved in 250 μL DMSO to give the appropriate sample solutions.

Strains for chemical investigations

Fungal strains investigated chemically were isolated as described above, and were deposited in the fungal culture collection of the research group of Prof. Dr. G. M. König and Dr. A. D. Wright, University of Bonn, Germany.

Strain N4-2 was isolated from the green alga *Ulva* sp. (Tönning, North Sea, Germany), and identified as *Ascochyta salicorniae*.

Strain M5 T2-1 was isolated from the red alga *Liagora viscida* (Forskkal) C. Agardh (Moraira, Mediterranean Sea, Spain), and identified as *Drechslera dematioidea*.

Strain N7-8 was isolated from the jellyfish *Aurelia aurita* (Tönning, North Sea, Germany), and identified as *Epicoccum purpurascens*.

Strain AZ14-4 was isolated from the brown alga *Fucus spiralis* (Azores, Atlantic Ocean, Portugal), and identified as *Phoma tropica*.

Strain M1-7-1 was isolated from the brown alga *Cladostephus spongiosus* (Moraira, Mediterranean Sea, Spain), and identified as *Sporormiella* species.

Strain CUX3-6 was isolated from the brown alga *Fucus vesiculosus* (Cuxhaven, North Sea, Germany), and identified as a *Myrioconium* species.

Strain N7-4 was isolated from the jellyfish *Aurelia aurita* (Cuxhaven, North Sea, Germany), and identified as *Stemphylium* species.

Strains were cultured in penicillium or Fernbach flasks containing 0.5 or 0.25 L solid medium respectively. Liquid cultures were grown in 1 L Erlenmeyer flasks with indentations containing 0.5 L medium, and shaken on an Infors Novotron rotary shaker at 65 rpm. Media were inoculated with small mycelium plugs from stock culture (liquid media) or a suspension of mycelium from small scale cultivation (one petri dish per five penicillium flasks) in sterilised water (solid media).

Media used for cultivation were biomalt agar with or without ASW added and malt extract Soya meal (MS) agar. If not stated otherwise, solid media contained 6.8 g/L (biomalt agar) and 7.6 g/L (MS agar) agar; solid media with ASW contained 80 % and liquid media 100 % ASW.

The isolate of *Ascochyta salicorniae* (N4-2) was cultured on eight litres of solid medium (biomalt with 80 % ASW) RT for 40 days.

The isolate of *Drechslera dematioidea* (M5 T2-1) was cultured on 13.5 litres of solid medium (biomalt without any salts added) at RT for 30 days.

The isolate of *Epicoccum purpurascens* (N7-8) was cultured on 4.5 l solid medium (malt extract Soya meal with 80 % ASW) at RT for 5 weeks.

The isolate of *Phoma tropica* (AZ14-4) was cultured on 14 l liquid medium (malt extract Soya meal with 100 % ASW) at RT for six weeks.

The isolate of *Sporormiella* sp. (M1-7-1) was cultured on 10 l solid medium (biomalt without any salts added) at RT for ten weeks.

The isolate of *Myrioconium* sp. (CUX3-6) was cultured on 10 l liquid medium (biomalt with 100 % ASW) at RT for six weeks.

The isolate of *Stemphylium* sp. (N7-4) was cultured on 10 l liquid medium (biomalt with 100 % ASW) at RT for three weeks.

If not stated otherwise, medium and mycelium were homogenised together using a Waring blender. To cultures on solid medium, 500 mL water were added to 1 L medium prior to homogenisation. Resultant mixtures were subsequently extracted with EtOAc (3 × 500 mL per 500 mL medium) to yield crude extracts.

3.5 Biological testing

Agar diffusion assays

Agar diffusion assays were carried out in principle according to Schulz *et al.* (1995). Test organisms were the bacteria *Bacillus megaterium* de Bary (gram positive) and *Escherichia coli* (Migula) Castellani & Chambers (gram negative), the fungi *Microbotryum violaceum* (Pers.) Roussel (Ustomycetes), *Mycotypha microspora* Fenner (Zygomycetes), *Eurotium repens* Corda (Ascomycetes) and *Fusarium oxysporum* Schltdl. (mitosporic fungi), and the alga *Chlorella fusca* Shih Krauss (Chlorophyceae).

Sample solutions contained 5 mg/mL extract or 1 mg/mL pure compound. Samples were prepared by taking 50 µL of each solution and pipetting it onto a sterile antibiotic filter disk (Schleicher & Schuell 2668), which was then placed onto the appropriate agar medium and sprayed with a suspension of the test organism. Growth media, preparation of spraying suspensions, and conditions of incubation were as employed by Schulz *et al.*, (1995). The radii of the resultant zones of inhibition were measured from the edge of the filter disks. For extracts, a growth

inhibition zone or complete inhibition zone ≥ 3 mm was regarded as a positive result; growth inhibition: growth of the appropriate test organism was significantly inhibited compared to a negative control; complete inhibition: no growth at all in the appropriate zone.

Tyrosine kinase inhibitory activity

The DMSO sample solution of the appropriate extract/pure compound was diluted with H_2O (1:1 v/v) to yield corresponding sample solutions (1 mg/mL). Pure compounds, if active at 200 µg/mL, were tested at various lower concentrations.

TK inhibitory activity was determined using a commercial test kit (Tyrosine Kinase Assay Kit, non-radioactively, Boehringer Mannheim, Cat. No. 1 534 513), modified by Dr. G. F. Matthée (Dissertation, 1999). Assays were carried out by either Dr. G. F. Kirsch or Ms. I. Rahaus using T cell tyrosine kinase $p56^{lck}$ (Upstate Biotechnology). Sample solutions were incubated with 1 µM TK substrate II, biotin-labelled (Boehringer Mannheim), 1 mM ATP, 10 mM $MgCl_2$, 1 U TK $p56^{lck}$, 20 µL dilution buffer, and 20 µL assay buffer for 1 h at 30 °C. The resultant concentration of the extract/compound in the test mixture was 200 µg/mL. Dilution buffer (pH 7.0) contained 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES), 10 % glycerol, and 0.1 % ethylphenolpoly(ethylene-glycolether)$_n$ (NP-40). Assay buffer (pH 7.5) contained 250 mM Tris, 25 mM NaF, 2.5 mM EDTA-Na_2, 4.0 mM [ethylenebis(oxyethylenenitrilo)]tetraacetic acid (EGTA), 5 mM DTT, and 150 µM Na_3VO_4. The enzyme activity was determined as described in the TK Assay Kit, except the measuring wavelength was 415 nm instead of 405 nm. In each test series 3 mM piceatannol (Boehringer Mannheim) was included as a positive control. Samples which reduced the enzyme activity to 60 % or less relative to a negative control were regarded as active.

HIV-1 reverse transcriptase inhibitory activity

The assay was performed by Mr. C. Dreikorn according to a protocol established by Dr. G. F. Kirsch. DMSO standard solutions of the appropriate extracts/compounds were diluted with lysis buffer (1:10 v/v) to yield corresponding sample solutions.

HIV-1 RT inhibitory activity was measured using 20 µL of the sample solution, 20 µL lysis buffer, and 20 µL reaction mixture which were then incubated for 1 h at 37 °C. The resultant concentration of the extract in the test mixture was 66 µg/mL. Lysis buffer (pH 7.8) contained 1 ng recombinant HIV-1 RT (Boehringer Mannheim), 50 mM Tris, 80 mM KCl, 2.5 mM dithiothreitol (DTT), 0.75 mM ethylendiamintetra-acetic acid (EDTA), and 0.5 % Triton-X100. The reaction mixture contained template/primer hybrid poly(A)*oligo(dT)15 (600A260nm/mL), 8 µM thymidine-5'-triphosphate (dTTP), 8 µM digoxigenin- and biotin-labeled-2'-deoxy-(uridine-5'-triphosphate) (dUTP), 40 µM TrisHCl, 230 mM KCl, 24 mM $MgCl_2$, and 8 mM DTT (all Boehringer Mannheim).

Enzyme activity was measured following the ELISA protocol described by Eberle & Seibl (1992) at λ = 415 nm versus λ = 490 nm using an ELISA reader (Rainbow, SLT Labinstruments Deutschland GmbH). As a positive control, 10 µM of phosphonoformic acid (Sigma) was included in each test series. Extracts which reduced the enzyme activity to 80 % or less relative to a negative control were regarded as active.

Antiplasmodial activity

Antimalarial activity of most pure compounds was determined by Dr. R. Kaminsky, Swiss Tropical Institute, Basel, Switzerland. The compounds were tested by a variation of the semiautomated microdilution assay against intraerythrocytic forms of *P. falciparum* derived from asynchronous stock cultures (Desjardins *et al.*, 1979). The reference strains used were K1 (Thailand; resistant to chloroquine and pyrimethamine) and NF 54 (an airport strain of unknown origin, susceptible to standard antimalarials) as described by Ridley *et al.* (1996).

Antitrypanosomal and cytotoxic activity

Activity against hemoflagellates which cause human sleeping sickness (*Trypanosoma brucei* subsp. *rhodesiense*) and Chagas disease (*Trypanosoma cruzi*) and also cytotoxic activity against rat skeletal muscle myoblast (L-6) cells and mouse peritoneal macrophages were assessed as described by Kaminsky and Brun (1998).

Inhibition of *Mycobacterium tuberculosis*

Activity of some pure compounds against *M. tuberculosis* $H_{37}Rv$ (ATCC 7294) was tested in cooperation with the research group of Prof. S. Franzblau, U.S. Department of Health and Human Services, GWL Hansen's Disease Center, Baton Rouge, Louisiana, USA, in the BATEC 460 system (Collins & Franzblau, 1997). Percent inhibition was calculated as [1-(growth index of test sample/growth index of control)] × 100.

Activity against the brine shrimp *Artemia salina* and the nematode *Caenorrhabditis elegans*

The assay used in this study was modified by Mr. L. Peters, Institute for Pharmaceutical Biology, University Bonn, Germany, and combines the investigation of activities against *A. salina* and *C. elegans*. Brine shrimps have been previously utilized in various bio-assay systems (Meyer *et al.*, 1982; Solis *et al.*, 1993).

Sample solutions contained 5 mg/mL extract or 1 mg/mL pure compound (in acetone:MeOH, 1:1). Samples were prepared by taking 5 µL of each solution and pipetting it into a vial of a sterile microtiter plate. Samples were dried by air and 125 µL assay medium were added to each well. This mixture was shaken for 10 min at RT. Then 50 µL of the *Artemia* culture medium together with a few *A. salina* larvae was added, followed by some *C. elegans* which were put to the border of each well by a spatula. They were washed into the wells by adding 125 µL assay medium. After an incubation period at RT the evaluation followed visually after 24 and after 48 h.

Assay medium: 0.1 g lecithin from frog eggs (Merck), 2.52 g $NaHCO_3$, 2.23 g KCl, 4.41 g $CaCl_2 \cdot 2H_2O$, 7.39 g $MgSO_4 \cdot 7H_2O$, 1000 mL aqua dest.

Culture medium for *A. salina*: 2.4 g Tris, 25.7 g NaCl, 4.7 g $MgCl_2$ (presolved), 0.7 g KCl, 0.2 g Na_2CO_3 (presolved), 6.4 g $MgSO_4$ (presolved), 1.1 g $CaCl_2$ (presolved), 1000 mL aqua (bidest.), adjusted to pH 7.1, stored at 4 °C.

Culture: 1 g *Artemia* cysts/400 mL culture medium (RT) in a 1 L beaker covered with gaze were ventilated under oxygen for 48 h. Medium was changed every day.

Collecting: The beaker was covered to the last 2 cm to the ground and illuminated with cold light. The supernatant were thrown away and the residue was put in a special basin. The adult were collected by illumination of only a little circle for nearly 2 h.

The nematode *Caenorrhabditis elegans* was cultured in petri dishes being inoculated with *E. coli*, and incubated at 25 °C for 24 h. Small pieces from a *C. elegans* culture had to be transferred to such a petri dish. Incubation at RT and at daylight.

Agar medium for *C. elegans*:

Solution A: 1.5 g NaCl, 8.5 g Bacto-agar (Sigma, A-7002), 2.5 g Bacto-peptone (Difco, 0118.01-8), 5.0 g yeast extract (Difco, 0127-01-7), 500 mL aqua bidest.

Solution B: 50 mg cholesterol (Sigma, C-8253), 10 mL EtOH (must not be autoclaved in contrast to solutions A, C-E).

Solution C: 1.39 g $CaCl_2$, 10 mL aqua bidest.

Solution D: 3.08 g $MgSO_4 \times 7H_2O$, 10 mL aqua bidest.

Solution E: 13.54 g KH_2PO_4, 4.45 g K_2HPO_4, 100 mL aqua bidest.

Solutions A-E were stored at 4 °C.

A mixture of 500 mL sol. A, 0.5 mL sol. B, 0.25 mL sol. C, 0.5 mL sol. D, and 12.5 mL sol. E was poured into petri dishes at nearly 50 °C.
Culture medium for *E. coli*: 10.0 g Bacto-Trypton (Difco, 0123-01-1), 2.5 g NaCl, 5.0 mg Uracil (Sigma, U-0750), 500 mL aqua bidest. (pH 7.4).

3.6 Chemicals

Media components:

Agar (Fluka 05040)
Biomalt (Villa Natura, Kirn)
Malt extract (Merck 5391)
Peptone from casein, tryptic digest (Fluka 70172)
Peptone from meat, enzymatic digest (Fluka 70175)
Peptone from Soya meal, papain-digested (Merck 7212)
Yeast extract (Fluka 70161)
Benzylpenicillin (Fluka 13750)
Streptomycin sulphate (Fluka 85880)
Carrots, potatoes and cornmeal were supplied by local food stores.
Water used was de-ionised using a Millipore (milli-Q®) system.
All other chemicals/components were research grade, and if not stated otherwise in the text, supplied by Merck.

Solvents:

EtOH for UV-measurements was from Merck (Uvasol® 980). All other solvents were research grade and supplied by Merck, except *n*-BuOH (Roth). Water used was deionised using a Millipore (milli-Q®) system. Acetone, $CHCl_3$, DCM, EtOAc, hexane, cyclohexane, MeOH and petroleum ether were distilled prior to use.
Acetone-d_6 (Chemotrade 97509, 99.8 % D)
Chloroform-d (Chemotrade, 99.87 % D)
Methanol-d_4 (ICB 0844-25, 99.8 % D)

Annotation

As mentioned above, parts of the current study have been published in advance. The following chapter, description of partial projects, is thus made up of these shortened papers (4.2 - 4.6), supplemented by a report on the isolation of fungi and the results of the performed screening programme (4.1).

Only four of the seven fungi (see 3.4) investigated for their secondary metabolite content in a large scale showed to produce these compounds in a minimum amount which is necessary for structure elucidation. This is the reason why nothing is reported about the natural product content of the investigated fungi *Sporormiella* sp., *Myrioconium* sp. and *Stemphylium* species.

4 Description of partial projects

4.1 Isolation and biological activities of fungi from marine sources - mainly algae

Introduction

The main goal of this study was the isolation of endophytic fungi from marine algae in order to investigate them for the production of new and biologically active metabolites.

The definition "fungal endophytes" includes all organisms inhabiting plant hosts at some time in their life and colonise internal plant tissues without causing apparent harm to their host (Schulz *et al.*, 1998). This includes also fungi having a more or less epiphytic phase and also latent pathogens (Petrini, 1991). Often endophytes remain asymptomatic for many years and only become parasitic when their hosts are stressed. For the isolation of endophytes it is important that collected algal material looks healthy, it must not have any visual symptoms of disease.

Fungal endophytes have to deal with their host plants. Often they develop chemical strategies which are important for their existence. This may be an explanation for the results of an investigation on the antialgal/herbicidal activity of fungal extracts by Schulz *et al.* (1999a). This study showed the number of active endophytic isolates to be three times higher than the number of active soil fungi and twice higher than the number of phytopathogens tested. As only few results on fungi associated with algae are available (König & Wright, 1996) the current study examined to which extent algae contain fungal endophytes, which genera of fungi are present, and what kind of metabolites they produce. Extracts of selected isolates are screened for their biological activities in order to find strains producing new biologically active secondary metabolites.

In order to ensure fungal isolates to be endophytic when obtained by an indirect isolation method (Kohlmeyer & Kohlmeyer, 1979), a surface sterilization of algal

material had to be performed. Algae with thin thalli are more sensitive towards sterilization agents than sturdy algae. Thus, a series of surface sterilization tests with EtOH (70 %) and sodium hypochlorite (2 %) was done for each alga. This secured for every investigated alga that fungal epiphytes were destroyed by the agent, but the inner algal tissue was not damaged as it may contain endophytic fungi. Effectiveness of surface sterilization was checked by making an imprint of the treated tissue on an biomalt medium agar plate. If the surface sterilization was successful no fungal colonies developed on the imprint medium.

As many endophytes, similar to other marine fungi, were considered to be slow growing (Siegel *et al.*, 1987) they are in danger of being overgrown by fast growing fungi. Thus, they will not be detected and isolated. This happens especially on nutrient rich media. Therefore, selective isolation media were used simulating the natural environment of the fungi to be isolated. Preferably, medium containing only the shredded algal tissue as nutrient was used. Alternatively, nutrient poor media such as SNA and GPY or agar only containing real sea water were prepared for this purpose.

Materials and methods (general procedures), see 3.3 and 3.4.

Results and Discussion

From 56 algal samples, 6 marine plants or plants from the intertidal zone, one sponge and one jellyfish a total of 428 fungal strains was isolated. Due to the great number of isolates, in most cases, only the genus was determined. A different morphology on the same growth medium suggested the isolates of many genera to represent several species. The results concerning the genera obtained and the number of isolates from each sample are given in Table 3.

The isolated fungi were identified as belonging to 6 genera of Ascomycetes, 2 genera of Zygomycetes and 37 genera of Mitosporic fungi; 110 strains remained sterile even though diverse media and conditions of culture were used to induce sporulation. The three obligate marine fungi (Kohlmeyer & Volkmann-Kohlmeyer, 1991) isolated were, *Ascochyta salicorniae* from the green alga *Ulva* sp. from

Tönning, Germany, North Sea, *Corollospora maritima* from the spermatophyta *Posidonia oceanica*, Moraira, Spain, Mediterranean Sea, and *Dendryphiella salina* from *Fucus vesiculosus*, *Salicornia europaea*, *Limonium vulgare* and *Amophila arenaria*, all derived from Tönning, Germany, North Sea. As many fungal genera, found by Kohlmeyer & Kohlmeyer (1979) being associated with algae, are composed of obligate marine species it is surprising that only three obligate marine isolates were found. Since some genera of mitosporic fungi, e.g., *Alternaria*, *Cladosporium*, *Coniothyrium* and *Phoma* are known to have marine representatives (Kohlmeyer & Volkmann-Kohlmeyer, 1991), it is possible that the real number of obligate marine fungi obtained was higher.

Some genera of fungi were found exclusively in samples from only one collection site (Table 3), e.g., fungi of the genus *Dendryphiella* were exclusively isolated from samples from Tönning, North Sea, and those of the genus *Myrioconium* from algal samples from Cuxhaven, North Sea. In accordance with the results of Höller, dealing with the isolation of fungi from sponges (dissertation, 1999), fungi of the genera *Acremonium*, *Cladosporium* and *Fusarium* were isolated from algae from almost every location. In contrast to the study of Höller, only two isolates of *Aspergillus* and two isolates of *Penicillium* were obtained. This possibly resulted from the fact that algae investigated were surface sterilised. Fungi of other genera, e.g., *Ascochyta*, *Chaetomella*, *Preussia* and *Sirococcus*, could be obtained exclusively from single algal samples. The greatest number of isolates (> 25) or the greatest diversity of genera (≥ 8) was obtained from the samples CUX1, CUX3, N1 and M7. Remarkably, algal samples collected from the North Sea (Cuxhaven and Tönning) contained most fungal isolates whereas from most algae collected at Spitzbergen, Arctic, hardly any fungal strains could be isolated. This result might correlate to water pollution caused by human beings.

These results contradict reports from literature where most fungi being associated with marine algae belong to the Ascomycetes and only few to the Deuteromycetes (mitosporic fungi) (Kohlmeyer & Kohlmeyer, 1979), since in the current study mainly mitosporic fungi could be isolated from algae.

Genera described by Kohlmeyer being parasitic on marine algae were not isolated during this study. When collecting algal samples we watched out for healthy looking plants because often changes of the outer appearance of the alga are caused by parasitic fungi. Our main focus was to isolate endophytic (and not parasitic) fungi as they are supposed to have the most creative secondary metabolism.

During the current study many fungal isolates obtained were before described to be saprobic fungi on marine algae, e.g., *Corollospora maritima* from *Fucus* sp., *Dendryphiella salina* from *Laminaria*, *Epicoccum* sp. from *Laminaria* sp., *Phoma* spp. from *Fucus vesiculosus*, *Stemphylium* sp. from *Sargassum* species (Kohlmeyer & Kohlmeyer, 1979). Thus, our results are surprising as only living algal samples were collected and surface sterilized.

Finally, Kohlmeyer & Kohlmeyer (1979) describe Phaeophyta to be the main substrate for saprobic fungi, probably because the tough brown algae are more resistant to decay than Chlorophyta and Rhodophyta. This would permit slow growing filamentous fungi to form mycelia and propagules, whereas the more delicate green and red algae are decomposed by bacteria and yeasts before the filamentous fungi have time to reproduce. By analogy with these data, in the current study brown algae were also a valuable source for filamentous fungi being compared to red and green algae (see Table 3).

Table 3. Genera and total number of fungal isolates obtained from marine organisms.

Sample / Fungal genera	*Fucus vesiculosus* (N-1)	*Salicornia europaea* (N-2)	*Enteromorpha* sp. (N-3)	*Ulva* sp. (N-4)	*Limonium vulgare* (N-5)	*Amophila arenaria* (N-6)	*Aurelia aurita* (N-7)	*Laminaria* sp. (CUX-1)	*Porphyra* sp. (CUX-2)	*Fucus vesiculosus* (CUX-3)	*Delesseria sanguinea* (HE-1)	*Petalonia fascia* (HE-3)	*Fucus vesiculosus* (WI-2)	*Enteromorpha* sp. (WI-4)	*Cladophora* sp. (SY-1)
a) Ascomycetes															
Chaetomium	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-
Corollospora	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Emericellopsis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Nectria	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Preussia	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sporormiella	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-
b) Zygomycetes															
Mucor	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Zygorhynchus	-	-	-	-	-	-	-	-	3	-	-	-	-	-	-
c) Mitosporic fungi															
Acremonium	1	-	-	-	-	-	-	1	-	1	-	1	-	3	-
Alternaria	1	-	3	-	-	2	1	-	-	1	-	-	-	-	-
Arthrinium	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ascochyta	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-
Aspergillus	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-
Aureobasidium	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Bispora	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-
Botrytis	1	-	-	-	-	-	-	-	-	-	-	-	4	-	-
Chaetomella	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Chrysosporium	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cladosporium	1	1	-	-	-	1	2	-	-	-	-	-	-	-	-
Cylindrocarpon	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-
Dendryphiella	7	5	-	-	2	1	-	-	-	-	-	-	-	-	-
Drechslera	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-
Epicoccum	1	-	-	-	-	-	1	-	-	-	-	-	1	-	-
Exophiala	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Fusarium	-	-	-	1	1	-	-	4	-	5	-	-	-	17	-
Geomyces	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Geotrichum	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gliocladium	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 3 continued.

Sample **Fungal genera**	***Fucus vesiculosus* (N-1)**	***Salicornia europaea* (N-2)**	***Enteromorpha* sp. (N-3)**	***Ulva* sp. (N-4)**	***Limonium vulgare* (N-5)**	***Amophila arenaria* (N-6)**	***Aurelia aurita* (N-7)**	***Laminaria* sp. (CUX-1)**	***Porphyra* sp. (CUX-2)**	***Fucus vesiculosus* (CUX-3)**	***Delesseria sanguinea* (HE-1)**	***Petalonia fascia* (HE-3)**	***Fucus vesiculosus* (WI-2)**	***Enteromorpha* sp. *(WI-4)***	***Cladophora* sp. (SY-1)**
Gliomastix	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Humicola	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Myrioconium	-	-	-	-	-	-	-	3	-	5	-	-	-	-	-
Nigrospora	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Paecilomyces	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-
Penicillium	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-
Pestalotiopsis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Phialophora	-	-	-	-	-	-	-	16	-	8	-	-	-	1	-
Phoma	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Scolecobasidium	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Scopulariopsis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sirococcus	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Stachybotrys	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Stemphylium	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-
Verticillium	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Wardomyces	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Xylaria	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mycelia sterilia	4	5	3	1	1	4	3	11	2	4	-	-	2	3	6
Total number	**17**	**11**	**6**	**4**	**4**	**8**	**8**	**35**	**5**	**26**	**1**	**1**	**11**	**24**	**6**

Table 3 continued.

Sample / Fungal genera	*Enteromorpha* sp. (SY-2)	*Enteromorpha* sp. (SY-3)	unidentified alga (SY-4)	*Petalonia zosterifolia* (OS-1)	*Enteromorpha* sp. (OS-2)	*Fucus vesiculosus* (OS-3)	*Enteromorpha* sp. (OS-4)	*Posidonia oceanica* (S-1)	*Posidonia oceanica* (S-2)	roots of *P. oceanica* (S-5)	*Cladostephus spongiosus* (M-1)	*Cystoseira* sp. (M-3)	*Acetabularia acetabulum* (M-4)	*Liagora viscida* (M-5)	*Herposiphonia* sp. (M-6)
a) Ascomycetes															
Chaetomium	-	-	-	-	-	-	1	-	-	1	-	-	-	1	1
Corollospora	-	-	-	-	-	-	-	2	3	-	-	-	-	-	-
Emericellopsis	-	-	-	-	-	-	-	-	-	-	1	-	1	-	-
Nectria	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Preussia	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sporormiella	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
b) Zygomycetes															
Mucor	-	-	-	-	4	-	-	-	-	-	-	-	-	-	-
Zygorhynchus	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
c) Mitosporic fungi															
Acremonium	-	-	-	1	-	1	-	-	-	2	-	-	-	-	-
Alternaria	-	-	-	-	-	-	-	3	-	-	-	1	1	5	-
Arthrinium	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ascochyta	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Aspergillus	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Aureobasidium	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-
Bispora	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Botrytis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Chaetomella	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Chrysosporium	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-
Cladosporium	-	1	-	2	-	1	4	3	-	-	1	-	-	1	-
Cylindrocarpon	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Dendryphiella	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Drechslera	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-
Epicoccum	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Exophiala	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-
Fusarium	-	-	-	5	1	1	4	-	1	1	-	-	-	-	1
Geomyces	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-
Geotrichum	-	-	-	-	1	-	-	-	1	1	-	-	-	-	-
Gliocladium	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-

Table 3 continued.

Sample / Fungal genera	*Enteromorpha* sp. (SY-2)	*Enteromorpha* sp. (SY-3)	unidentified alga (SY-4)	*Petalonia zosterifolia* (OS-1)	*Enteromorpha* sp. (OS-2)	*Fucus vesiculosus* (OS-3)	*Enteromorpha* sp. (OS-4)	*Posidonia oceanica* (S-1)	*Posidonia oceanica* (S-2)	roots of *P. oceanica* (S-5)	*Cladostephus spongiosus* (M-1)	*Cystoseira* sp. (M-3)	*Acetabularia acetabulum* (M-4)	*Liagora viscida* (M-5)	*Herposiphonia* sp. (M-6)
Gliomastix	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-
Humicola	-	-	-	-	-	-	-	-	-	-	2	-	-	-	-
Myrioconium	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Nigrospora	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Paecilomyces	-	-	-	-	-	2	-	-	-	-	-	-	-	-	-
Penicillium	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Pestalotiopsis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Phialophora	14	2	-	1	-	-	-	-	-	-	-	-	-	-	-
Phoma	2	-	-	1	-	-	-	-	-	-	-	-	-	-	-
Scolecobasidium	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-
Scopulariopsis	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-
Sirococcus	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Stachybotrys	-	-	-	-	-	-	-	-	-	-	3	-	-	-	-
Stemphylium	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-
Verticillium	-	-	-	-	-	1	-	-	1	-	-	-	-	-	-
Wardomyces	-	5	-	-	-	-	1	-	-	-	-	-	-	-	-
Xylaria	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mycelia sterilia	1	-	2	8	1	1	6	1	4	-	5	1	-	3	4
Total number	**17**	**8**	**2**	**18**	**7**	**8**	**18**	**9**	**12**	**7**	**13**	**3**	**2**	**11**	**6**

Table 3 continued.

Sample / Fungal genera	*Jania rubens* (M-7)	unidentified alga (M-8)	unidentified alga (G-1)	*Stypopodium* sp. (G-2)	*Osmundia pinnatifida* (AZ-1)	*Valomia utricularis* (AZ-3)	*P.* cf. *fucoides* sp. (AZ-4)	*Cystoseira foeniculaceus* (AZ-5)	*Dictyota* sp. (AZ-6)	*Thedania anhelaus* (AZ-7)	*Pterocladia capillacea* (AZ-8)	*Corallina elongata* (AZ-9)	*Enteromorpha* sp. (AZ-10)	*Sargassum* sp. (AZ-11)	*Cystoseira foeniculaceus* (AZ-12)
a) Ascomycetes															
Chaetomium	2	2	-	-	-	3	-	-	2	-	-	-	-	-	-
Corollospora	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Emericellopsis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Nectria	-	-	-	-	-	-	3	-	-	-	-	-	-	-	-
Preussia	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-
Sporormiella	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-
b) Zygomycetes															
Mucor	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Zygorhynchus	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
c) Mitosporic fungi															
Acremonium	-	-	-	1	-	1	-	-	-	1	-	-	-	-	-
Alternaria	6	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Arthrinium	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ascochyta	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Aspergillus	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-
Aureobasidium	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Bispora	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Botrytis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Chaetomella	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-
Chrysosporium	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cladosporium	3	-	-	-	5	-	1	-	-	-	-	-	-	-	-
Cylindrocarpon	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-
Dendryphiella	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Drechslera	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Epicoccum	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-
Exophiala	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Fusarium	1	1	-	-	-	2	5	2	1	2	2	-	-	2	-
Geomyces	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
Geotrichum	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-
Gliocladium	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 3 continued.

Sample / Fungal genera	***Jania rubens* (M-7)**	**unidentified alga (M-8)**	**unidentified alga (G-1)**	***Stypopodium* sp. (G-2)**	***Osmundia pinnatifida* (AZ-1)**	***Valomia utricularis* (AZ-3)**	***P.* cf. *fucoides* sp. (AZ-4)**	***Cystoseira foeniculaceus* (AZ-5)**	***Dictyota* sp. (AZ-6)**	***Thedania anhelaus* (AZ-7)**	***Pterocladia capillacea* (AZ-8)**	***Corallina elongata* (AZ-9)**	***Enteromorpha* sp. (AZ-10)**	***Sargassum* sp. (AZ-11)**	***Cystoseira foeniculaceus* (AZ-12**
Gliomastix	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-
Humicola	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Myrioconium	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Nigrospora	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-
Paecilomyces	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-
Penicillium	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Pestalotiopsis	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-
Phialophora	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Phoma	-	-	-	2	-	6	-	1	-	-	-	-	2	-	-
Scolecobasidium	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Scopulariopsis	-	-	-	-	-	-	-	-	-	3	-	-	-	-	-
Sirococcus	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Stachybotrys	-	2	1	1	-	-	-	-	-	-	-	-	-	-	-
Stemphylium	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Verticillium	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Wardomyces	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-
Xylaria	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mycelia sterilia	4	5	-	2	2	2	-	1	-	-	1	-	-	2	1
Total number	**20**	**12**	**1**	**8**	**7**	**15**	**10**	**6**	**4**	**8**	**3**	**1**	**2**	**4**	**2**

Table 3 continued.

Sample / Fungal genera	*Fucus spiralis* (AZ-14)	*Laminaria digitata* (ARKT-1)	*Laminaria sacchorhiza* (ARKT-3)	Total number
a) Ascomycetes				
Chaetomium	-	-	-	**14**
Corollospora	-	-	-	**5**
Emericellopsis	-	-	-	**2**
Nectria	-	-	-	**3**
Preussia	-	-	-	**1**
Sporormiella	-	-	-	**2**
b) Zygomycetes				
Mucor	-	-	-	**4**
Zygorhynchus	-	-	-	**3**
c) Mitosporic fungi				
Acremonium	1	-	-	**15**
Alternaria	-	-	-	**24**
Arthrinium	-	-	-	**1**
Ascochyta	-	-	-	**1**
Aspergillus	-	-	-	**2**
Aureobasidium	-	-	-	**1**
Bispora	-	-	-	**1**
Botrytis	-	-	-	**5**
Chaetomella	-	-	-	**1**
Chrysosporium	-	-	-	**1**
Cladosporium	-	1	-	**28**
Cylindrocarpon	-	-	-	**2**
Dendryphiella	-	-	-	**15**
Drechslera	-	-	-	**2**
Epicoccum	1	-	-	**5**
Exophiala	-	-	-	**1**
Fusarium	-	-	-	**60**
Geomyces	-	-	-	**2**
Geotrichum	-	-	-	**4**
Gliocladium	-	-	-	**1**

Table 3 continued.

Sample / Fungal genera	*Fucus spiralis* (AZ-14)	*Laminaria digitata* (ARKT-1)	*Laminaria sacchorhiza* (ARKT-3)	Total number
Gliomastix	-	-	-	**2**
Humicola	-	-	-	**2**
Myrioconium	-	-	-	**8**
Nigrospora	-	-	-	**1**
Paecilomyces	-	-	-	**4**
Penicillium	-	-	1	**2**
Pestalotiopsis	-	-	-	**1**
Phialophora	-	1	7	**50**
Phoma	-	-	-	**14**
Scolecobasidium	-	-	-	**1**
Scopulariopsis	-	-	-	**4**
Sirococcus	-	-	-	**1**
Stachybotrys	-	-	-	**7**
Stemphylium	-	-	-	**4**
Verticillium	1	-	-	**3**
Wardomyces	-	-	-	**7**
Xylaria	-	-	-	**1**
Mycelia sterilia	4	-	-	**110**
Total number	**7**	**2**	**8**	**428**

Biological activity of extracts of selected fungal strains

Antimicrobial activity in agar diffusion assays

Due to the great number of isolated fungal strains, only extracts of some selected samples could be tested for their biological activities. Criteria for selection were the taxonomy of the isolates and the diversity of secondary metabolite chemistry of the respective genera, as found in the Chapman & Hall database. Strains of ubiquitous genera with well known secondary metabolism were not further investigated, in order to avoid the isolation of known metabolites. In contrast, all strains of Mycelia sterilia except eight which did not grow any more, were screened for their biological activities, as they might belong to rare and interesting genera. Thus, 265 strains were selected and grown on three different media, i.e. B, MS, and Bfl.

Cultures were extracted with EtOAc to yield a total of 795 extracts. In agar diffusion assays for antibacterial, antifungal and antialgal activity, 421 extracts (53.0 %) resulting from 222 strains (83.8 %) proved to be active (growth inhibition zone or complete inhibition zone ≥ 3 mm) against at least one test organism.

Table 4 provides the number of antimicrobially active fungal strains. Detailed results of the agar diffusion assays are listed in chapter 8.2.

Table 4. Number of antimicrobially active fungal strains assessed in agar diffusion assays.[1]

Genus	Number of strains tested	*E. c.*[2]	*B. m.*[2]	*M. v.*[2]	*E. r.*[2]	*F. o.*[2]	*M. m.*[2]	*C. f.*[2]	Total number of active strains
Alternaria	11	-[3]	9	4	11	-	1	3	**11**
Arthrinium	1	-	-	1	-	-	-	-	**1**
Ascochyta	1	-	1	-	1	-	1	-	**1**
Aureobasidium	1	-	1	1	-	1	-	-	**1**
Bispora	1	-	-	1	1	-	-	-	**1**
Chaetomella	1	-	-	1	-	-	-	-	**1**
Chaetomium	11	-	7	7	7	1	1	-	**10**
Chrysosporium	1	-	-	-	1	-	-	-	**1**
Corollospora	5	-	2	5	3	-	-	-	**5**
Cylindrocarpon	1	-	1	1	-	-	-	-	**1**
Dendryphiella	14	-	9	6	7	-	1	-	**12**
Drechslera	2	-	2	2	2	-	-	1	**2**
Emericellopsis	2	-	1	1	-	-	-	-	**2**
Epicoccum	2	-	1	-	1	-	-	-	**1**
Geomyces	2	-	2	1	-	-	-	-	**2**
Geotrichum	4	-	4	4	-	-	-	1	**4**
Gliocladium	1	-	1	1	1	-	-	-	**1**
Gliomastix	2	-	2	1	1	-	-	1	**2**
Humicola	2	-	1	2	-	-	-	-	**2**
Myrioconium	10	-	7	9	9	1	-	2	**10**
Nigrospora	1	-	-	1	1	-	-	-	**1**
Paecilomyces	5	-	3	1	2	-	1	-	**4**
Pestalotiopsis	1	-	-	1	1	-	-	-	**1**
Phialophora	42	-	16	18	13	1	-	6	**34**
Phoma	13	-	7	6	1	-	-	1	**11**
Preussia	1	-	1	-	-	-	-	-	**1**
Scolecobasidium	2	-	1	1	1	-	-	-	**1**
Scopulariopsis	4	-	1	4	2	1	-	1	**4**
Sporormiella	2	-	-	1	1	-	1	1	**1**
Stachybotrys	7	-	3	1	1	-	-	-	**4**
Stemphylium	1	1	2	2	1	-	-	-	**1**
Wardomyces	7	-	1	3	1	-	-	-	**3**
Xylaria	1	-	-	1	1	-	1	-	**1**
Zygorhynchus	1	-	1	1	-	-	-	-	**1**
Mycelia sterilia	102	3	51	60	52	5	8	7	**83**
Total number	**265**	**4**	**138**	**149**	**123**	**10**	**15**	**24**	**222**

[1] EtOAc extracts from fungal cultures on three different media were tested in agar diffusion assays at a concentration of 5 mg/mL (resolved in MeOH:acetone, 1:1). If one of the extracts was active, the corresponding strain was regarded as active.
[2] Test organisms: *E.c. Escherichia coli*, *B.m. Bacillus megaterium*, *M.v. Microbotryum violaceum*, *E.r. Eurotium repens*, *F.o. Fusarium oxysporum*, *M.m. Mycotypha microspora*, *C.f. Chlorella fusca*.
[3] Isolates whose extracts produced an inhibition zone or a complete inhibition zone ≥ 3 mm were regarded as active; - no such activity.

In agreement with Höller's results (dissertation, 1999), antifungal activity was the most common inhibitory effect: 70.7 % of the fungal strains tested exhibited antifungal properties for at least one extract. The sensitivity of fungi used as test organisms in the agar diffusion assays varied greatly. The growth of *E. repens* and *M. violaceum* was inhibited by extracts of 46.4 % of the tested strains, respectively, whereas *M. microspora* was inhibited by extracts of 5.7 %, and *F. oxysporum* by extracts of only 3.8 % of the tested strains.

Antibacterial activity was found for 53.6 % of extracts of the selected strains. Extracts of only four strains (1.5 %) were found to be active against the gram-negative bacterium *E. coli,* whereas extracts of 138 strains (52.1 %) were found to be active against the gram-positive bacterium *B. megaterium*. Extracts of 24 strains (9.1 %) were found to be active against the green alga *C. fusca*.

The largest number of strains investigated for antimicrobial activity belonged to the genus *Phialophora* (42 strains) or to the Mycelia sterilia (102 strains). The agar diffusion assays demonstrated that 34 strains of the genus *Phialophora*, and 83 of 102 tested strains of the Mycelia sterilia showed antimicrobial activity.

Extracts of 73 strains selectively inhibited only one test organism (Table 5). No fungal extract showed a broad spectrum of antimicrobial activity towards all test organisms. The extracts of most strains showed antimicrobial activity towards only some of the test organisms of each group or towards organisms of one or two groups of test organisms. Extracts from 86 strains were found to be both antibacterial and

antifungal, extracts from 6 strains were found to be antifungal and antialgal, extracts from 4 strains were found to be antibacterial and antialgal. The extracts of 14 strains inhibited members of all three groups of test organisms.

Table 5: Selectivity of the antimicrobial activity of culture extracts of marine-derived fungi in the agar diffusion assays.

Number of strains found to be exclusively active against:[1]	
B. megaterium	28 (10.4 %)
E. repens	18 (6.8 %)
M. microspora	1 (0.3 %)
M. violaceum	25 (9.3 %)
C. fusca	1 (0.3 %)
Number of strains found to be active against two or more groups of test organisms:	
antibacterial and antifungal	86 (32.5 %)
antifungal and antialgal	6 (2.3 %)
antibacterial and antialgal	4 (1.5 %)
antibacterial, antifungal and antialgal	14 (5.3 %)

[1] Extracts of none of the strains were found to be selectively active against *E. coli* or *F. oxysporum*; extracts which produced an inhibition zone or a complete inhibition zone with a radius ≥ 3 mm were regarded as active.

Inhibition of tyrosine kinase and HIV-1 reverse transcriptase

Inhibition of HIV-1 reverse transcriptase and tyrosine kinase was measured using ELISA based systems (Eberle & Seibl, 1992). Selection of strains to be tested was mainly based on the TLC profile and on the antimicrobial activity (growth inhibition zone > 10 mm) in the agar diffusion assays. These criteria for the choice of isolates assume that a strain is likely either to produce a large number of metabolites, some of which are active in one, and others in another assay, or that a metabolite is likely to be active in more than one assay system. Thus, 43 strains, representing 12 genera and 16 Mycelia sterilia were cultured and extracted for the enzyme assays. From the resulting 43 EtOAc extracts, six strains (14.0 %) were found to be active in both the HIV-1 RT and TK assay, 38 strains (88.4 %) in the TK assay (Table 6), no extract inhibiting HIV-1 RT selectively.

Table 6. Culture extracts (EtOAc) of marine-derived fungi and their activity towards HIV-1 reverse transcriptase and tyrosine kinase p56^{lck}.

Fungal strain (code number[1])	HIV-1 reverse transcriptase activity[2]	Tyrosine kinase p56^{lck} activity[2]
M 4-20-1	0.0	0.9
G 2-4-1	n.a.[3]	25.2
S 2-6	n.a.	37.0
CUX 3-14	61.3	0.5
CUX 3-6	n.a.	2.2
CUX 3-10-	52.4	0.3
CUX 3-3	74.6	2.4
CUX 1-9-2	n.a.	4.3
M 7-8-1	65.2	2.8
M 8 T1-2	n.a.	2.4
M 5-5-1	n.a.	0.5
OS 5-19-1	n.a.	0.0
AZ 7-13-3	n.a.	2.6
AZ 5-10-1	n.a.	1.9
AZ 6-1-1	n.a.	0.1
AZ 6-3	n.a.	0.6
S 1-2	n.a.	1.8
S 2-3	n.a.	47.1
M 1-7-1	n.a.	n.a.
M 1-13-1-2	n.a.	21.1
M 1-14-1	n.a.	n.a.
M 7-11-1	n.a.	0.3
G 2-1	n.a.	n.a.
M 1-13-1-1	n.a.	57.5
N 4-1	n.a.	7.1
N 7-8	n.a.	0.0
S 2-7	n.a.	14.9
S 2-8	n.a.	n.a.
S 2-13	n.a.	46.1
SY 4 T 5-1	n.a.	3.6
S 5-4	n.a.	17.4
WH 4 T 2-3	n.a.	6.0
G 2-4-1	n.a.	17.3
CUX 1 T 5	n.a.	23.8
CUX 1 T 5-1	n.a.	n.a.
CUX 1 T 5-2	n.a.	18.5
CUX 1-1-1	n.a.	0.0
CUX 1-3	n.a.	4.6
CUX 1-4	n.a.	2.8

Table 6 continued.

Fungal strain and code number[1]	HIV-1 reverse transcriptase activity[2]	Tyrosine kinase p56^{lck} activity[2]
CUX 3-1	n.a.	5.4
CUX 3-2	70.7	0.0
CUX 3-3	n.a.	1.5
CUX 3-15	n.a.	1.1
CUX 3-8-1	n.a.	7.0

[1]Taxonomy is described in chapter 8.2.

[2] Percentage of enzyme activity observed relative to a negative control (100 % HIV-1 RT or TK p56^{lck} activity; Lck = lymphocytic kinase). Reduction of enzyme activity to 80 % or less (HIV-1 RT), at a concentration of 66 µg/mL, or 60 % or less (TK p56^{lck}), at a concentration of 200 µg/mL, was regarded as a significant inhibition. Piceatannol (3 mM, rest activity of TK 2.3 %) and phosphonoformic acid (10 µM, rest activity of HIV-1 RT 23.5 %) were used as positive controls.

[3] n.a. = not active

Antiplasmodial, antitrypanosomal and cytotoxic activities

EtOAc extracts of nine selected strains were tested for inhibitory activity against two different strains of *Plasmodium falciparum*, *Trypanosoma* spp., and for cytotoxic effects towards L-6 (rat skeletal muscle myoblast cells) cells. One strain, a *Stemphylium* sp. (N7-4), exhibited significant activities against both *Trypanosoma* strains and cytotoxic effects against L-6 cells. Three strains showed weak antitrypanosomal activity. Two strains had weak antiplasmodial activity against *Plasmodium falciparum* K1, one against strain NF 54. Results are shown in Table 7.

Table 7. Activities of culture extracts from marine-derived fungi towards two strains of *Plasmodium falciparum*, two *Trypanosoma spp.,* and cytotoxicity against rat skeletal muscle myoblast L-6 cells.

Fungal strain (code)	K1 [1] [ng/mL]	NF54 [1] [ng/mL]	*T.b. rhodesiense* [2]	*T. cruzi* [2]	Cytotoxicity L-6 [2]
M.s. [3] S2-8 [4]	> 10000	> 10000	> 90	> 90	> 90
M.s. OS1-15-1 [4]	> 10000	> 10000	> 90	> 90	> 90
M.s. OS1-5-1-1 [4]	> 10000	> 10000	> 90	> 90	> 90
Stemphylium sp. N7-4 [5]	9186	8948	3.3 (IC_{50} = 0.38)	30 (IC_{50} = 3.0)	30
Drechslera dematioidea M5T2-1 [6]	9370	> 10000	90 (IC_{50} = 17.71)	90 (IC_{50} = 19.2)	90
Epicoccum purpurascens N7-8 [4]	> 10000	> 10000	90 (IC_{50} = 18.90)	90 (IC_{50} = 44.3)	90
Phoma tropica AZ14-4 [7]	> 10000	> 10000	> 90	> 90	> 90
M.s. OS1-5-1-2 [6]	> 10000	> 10000	> 90	> 90	> 90
Ascochyta salicorniae N4-2 [5]	> 10000	> 10000	> 90	90 (IC_{50} = 32.0)	90

[1] IC_{50} values are given. Antiplasmodial activity was measured against two reference strains of *Plasmodium falciparum* K1 (Thailand; resistant to chloroquine and pyrimethamine), and NF 54 (an airport strain of unknown origin; susceptible to standard antimalarials).
[2] Values are MIC [μg/mL].
[3] M.s. = Mycelia sterilia
[4] Solid malt extract Soya meal medium was used, supplied with 80 % ASW.
[5] Liquid biomalt medium was used, supplied with 100 % ASW.
[6] Solid biomalt medium was used, no salt was supplied.
[7] Liquid malt extract Soya meal medium was used, supplied with 100 % ASW.

4.2 Differences between marine and terrestrial *Phoma* species as determined by HPLC-DAD and HPLC-MS

Abstract

Ethyl acetate extracts of 26 terrestrial and 16 marine-derived *Phoma* spp. were analysed using RP-HPLC coupled with diode-array detection (DAD) and thermospray mass spectrometry (TSP-MS). For all samples, the retention times, UV and mass spectral data were determined. Twenty-two mass spectral data points from single fungal metabolites could be identified as being discriminative between the two groups of fungi and were analysed by regression analysis. The occurrence of these mass units allowed 84 % of all of the extracts to be confirmed as being derived from either the terrestrial or marine samples. This finding suggests that marine-derived *Phoma* spp. differ significantly from terrestrial *Phoma* spp. with respect to their secondary metabolite content. The number of detected secondary metabolites per strain, and the number of compounds per strain which were unique to the marine-derived *Phoma* spp. did not differ markedly from the findings with their terrestrial counterparts.

Introduction

Fungi have for many decades been a favoured source of interesting new natural products, mainly due to their highly developed and diverse secondary metabolism. Thus, terrestrial fungi have been intensively studied. In direct contrast, however, fungi from the marine environment are less well investigated. For this reason the scientific interest in natural products from marine-derived fungi has increased dramatically in recent years (König & Wright 1996, Pietra 1997).

As a results to these efforts, more than a hundred secondary metabolites from marine fungi have been described (Biabani & Laatsch, 1998). Among these are naphtoquinone polyketides such as obionin A, which shows CNS activity (Poch & Gloer, 1989b), diketopiperazines, e.g., leptosins A and C, with *in vivo* anti-tumour activity (Takahashi *et al.*, 1994a), terpenoids, e.g., isoculmorin (Alam *et al.*, 1996), and phomactins A-F, which are potent and specific PAF antagonists (Sugano *et al.*, 1991, 1994, 1995).

In an effort to isolate marine fungi associated with sponges and algae, and to investigate their secondary metabolite production, a collection of numerous strains belonging to the genus *Phoma* has been assembled. This genus contains many terrestrial fungi as well as obligate marine fungi, e.g., *Phoma laminariae* and *P. suaedae*. Coelomycetes (mitosporic fungi), which probably belong to the genus *Phoma*, are often isolated from substrates of the marine and estuarine environment (Kohlmeyer & Volkmann-Kohlmeyer, 1991), as well. Since a multitude of metabolites from terrestrial *Phoma* spp. have already been described, e.g., cytochalasins (Aldridge *et al.*, 1967; Capasso *et al.*, 1991; Evidente *et al.*, 1992) cavoxin, cavoxone (Evidente & Randazzo, 1985), cavoxinine and cavoxinone (Evidente, 1987), phomaligols and phomaligadiones (Soledade *et al.*, 1993), it seemed questionable whether marine *Phoma* spp. would yield new natural products. Thus, a study which compared the biosynthetic capabilities of marine-derived and terrestrial strains was embarked upon.

Various methods for the separation and detection of fungal metabolites have been described, e.g., TLC (Durackova *et al.*, 1976; Gorst-Allman & Steyn, 1979; Paterson,

1986), GC-MS (Rosen *et al.*, 1986; Krishnamurthy & Sarver 1986), HPLC with UV detection (Frisvad, 1987), HPLC with diode array detection (DAD; Frisvad & Thrane, 1987), HPLC with thermospray mass spectrometric (TSP-MS) detection (Rajakylä *et al.*, 1987), and HPLC with electrospray (ES) MS detection (Smedsgaard & Frisvad, 1996).

In the current investigation, ethyl acetate extracts of fungal cultures were separated by RP-HPLC, and fungal metabolites were detected using DAD and TSP-MS detection. The resultant data were then used to compare the metabolite content/pattern of extracts of terrestrial and marine-derived *Phoma* species. Interpretation of these results by regression and cluster analyses then allowed the predictability of affiliations of fungal strains to the marine or terrestrial group to be investigated. From this investigation it should then be possible to show whether there are indeed two distinct groups of fungi, and if marine-derived fungi differ from their terrestrial counterparts. Taking this into account it could be then speculated that marine-derived *Phoma* spp. most probably contain natural products not to be found in terrestrial *Phoma* species.

Material and methods

Fungal material

Marine-derived *Phoma* strains were provided by Dr. U. Höller. They originated from four sponge species (*Ectyplasia perox*, *Halichondria panicea*, *Myxilla incrustans*, and *Leucosolenia* sp.), all collected from the waters around Helgoland, North Sea, Germany, and from four algae, two collected around Helgoland, and the other two around Tenerife, Spain. Terrestrial *Phoma* spp. were provided by Dr. B. Schulz, and were obtained from soil samples (Kenya, Malaysia, Canada, Japan, U.S., Philippines and Germany). Endophytic *Phoma* spp. were isolated from the terrestrial plants *Anemone nemorosa*, *Vaccinium vitis-idaea*, *Larix* sp., *Carduus* sp., and *Pastinacea* sp., all collected near Braunschweig, Germany (Table 8).

Table 8. Origin of fungal isolates.

Terrestrial *Phoma* sp.	Marine-derived *Phoma* sp.
T 1-T 3 Kenya, soil	M 1 brown alga, Tenerife
T 4 Malaysia, soil	M 2 alga, Tenerife
T 5 Kenya, soil	M 3 brown alga, Tenerife
T 6 Canada, soil	M 4-M 7 *Ectyplasia perox*, sponge
T 7 Japan, soil	M 8 alga, Helgoland
T 8 Florida, soil	M 9 red alga, Helgoland
T 9 sewage plant, Othfresen, soil	M 10-M 13 *Halichondria panicea*, sponge
T 10 Heidbergsee, Germany, soil	M 14-M 20 *Myxilla incrustans*, sponge
T 11 *Anemone nemerosa*, endophyte	M 21-M25 *Leucosolenia* sp., sponge
T 12 *Vaccinium vitis-idaea*, endophyte	M 26 *Halichondria panicea*, sponge
T 13 Philippines, soil	
T 14 *Larix* sp., endophyte	
T 15 thistle, endophyte	
T 16 *Pastinacea*, endophyte	

T: terrestrial isolate

M: marine-derived isolate

For the isolation of fungal strains, the same methods were used as described by Höller *et al.* (1999) for marine samples, and Schulz *et al.* (1993) for terrestrial samples. In order to culture fungal strains, three different media were tested for their suitability, namely, biomalt agar (20 g biomalt, Villa Natura, Kirn, 6.8 g agar and 1000 mL demineralised water), malt extract Soya meal agar (30 g malt extract, Merck, 3 g peptone from Soya meal, papain-digest, Merck, 7.6 g agar, 800 mL artificial sea water and 200 mL demineralised water), and algal medium [30 g (fresh weight) blended *Fucus vesiculosus*, 10 g agar and 1 L artificial sea water]. Plates were incubated for 3 weeks at RT. For HPLC analysis, each strain was inoculated on 40 mL of each of the three media.

Extraction of fungal material

Fungal cultures were homogenised using an Ultra Turrax model T25 at 8000 $U \cdot min^{-1}$ and extracted three times with 50 mL EtOAc. Solvent was removed *in vacuo* at 35 °C on a rotary evaporator. Resultant extracts were dissolved in MeOH/H_2O (1:9) to give solutions with a final concentration of 5 mg/mL.

TLC analysis

For the TLC comparison of extracts, silica gel 60 F_{254} layers (Merck) were used with CH_2Cl_2:MeOH (95:5) as the mobile phase. Substances were detected at UV 254/366 nm and with vanillin/H_2SO_4/120 °C. Compounds resulting from the ingredients of the medium were not considered.

HPLC analysis

HPLC-DAD analysis

Eluent delivery was provided by a Merck Hitachi (Darmstadt, Germany) model L-6200 A HPLC pump equipped with a Rheodyne (Berkeley, CA, USA) injection valve with a 50 µL sample loop. UV spectra were recorded using a Merck Hitachi model L-4500 A DAD in the range 200-400 nm and data were processed with Hitachi D-7000 HPLC system manager software. Peaks with identical UV maxima but different retention times were considered to be the same compound if they were detected in the range of ±3 minutes.

HPLC-TSP-MS

The HPLC system consisted of a Beckman (Beckman Instruments, San Ramon, USA) model 116 solvent delivery module, a Gynkotek (Gynkotek, Germering, Germany) model M 250 B gradient controller, a model ERC-3512 degasser (ERMA, Alteglofsheim, Germany); and a Rheodyne 7125 injection valve with a 50 µL sample loop. The chromatograph was coupled to a TSP-1 interface with control module (Finnigan MAT, Bremen, Germany). The settings were: first mass, *m/z* 155; last mass, *m/z* 750; scan rate, 2 s per scan; SEV, 1200 V; vaporizer temperature, 85 °C; aerosol temperature, 280 °C. Only base peaks (100 % relative intensity) were used for further investigations. Peaks with the same mass but different retention times detected in the range of ±3 minutes were considered to be the same compound.

HPLC separations were performed using a LiChroCart RP-18 column (250 x 4 mm i.d.; 5 µm; Merck), equipped with a LiChroCart 4-4 LiChrospher 100 RP-18 pre-column (5 µm; Merck), eluted with a linear gradient from 100 % water (Milli-Q®-purified; Millipore-Waters, Millipore, Eschborn, Germany) to 100 % MeOH (HPLC grade; Merck) in 60 min followed by 100 % MeOH for 10 min, all at a flow-rate of 0.5 mL/min. An aliquot (50 µL) of dissolved fungal extract (9:1, H_2O:MeOH), containing 250 µg crude extract was injected. For the LC-TSP experiment Milli-Q®-water was added postcolumn (0.8 mL/min) in order to obtain a flow rate of 1.3 mL/min, which was necessary for the use of thermospray interfaces.

Statistical methods

The program "Statistica" from StatSoft (Tulsa, OK, USA), was used for regression and cluster analyses as well as for all other calculations.

Results and Discussion

The aim of this study was to investigate whether marine-derived *Phoma* spp. could be an interesting and, perhaps, unique source of new secondary metabolites, especially when compared to their terrestrial counterparts. In an attempt to reflect the diversity of flora and fauna existing on land and in the sea, *Phoma* spp. from many

different sources were investigated. A total of 42 fungal strains, all belonging to the genus *Phoma*, were obtained: 21 strains of marine origin were isolated from sponges, and five further *Phoma* strains were obtained from algae. Eleven terrestrial *Phoma* spp. were obtained from soil, and five endophytic *Phoma* spp. were isolated from terrestrial plants (Table 8).

Three different cultivation media were tested in order to find the most suitable one for the production of natural products. Two media were based on biomalt, and malt extract Soya meal, respectively. To the malt extract Soya meal medium, artificial sea water was added in order to adapt culture conditions to the natural environment of marine fungi. The third medium was supplemented with blended tissue of the brown alga *Fucus vesiculosus* as sole nutrient source. TLC comparison of culture extracts of all media revealed that the chosen *Phoma* spp. produced the highest number of metabolites on biomalt medium, as evidenced by the number of substances detected. Following this investigation, it was decided to cultivate and then analyse only extracts of fungi grown on biomalt agar without added salts.

In a first step, an appropriate HPLC separation with good resolution and reproducibility had to be established. Several mobile phases, e.g., mixtures of water and acetonitrile or methanol, addition of buffer (NH_4CH_3COO buffer), and different solvent gradients, were tested with reversed-phase (RP-18) column. It was found that RP-18 HPLC with a water:MeOH gradient (see Material and Methods) yielded the overall best results, and allowed all fungal culture extracts to be finger-printed. In most cases, chromatograms indicated reasonable metabolite separation with good reproducibility (Figs. 2 and 3). Eluted metabolites were detected using DAD and TSP-MS. As DAD is a specific detection method showing only substances with a significant UV-absorption in the selected wavelength range (200-400 nm), it was not surprising that the number of signals observed in DAD-based chromatograms was notably less than those detected with TSP-MS. For the latter detection method a discharge electrode was used which enabled most compounds to be ionised. Thus, TSP-MS detection provided the most information on the composition of the investigated fungal extracts.

Using HPLC-DAD for the detection of natural products in culture extracts of *Phoma* spp., an average of 5.4 metabolites were detected per strain in the terrestrial group [with a standard deviation (SD) of 3.7]. A similar number, 4.2 metabolites per strain (SD 2.8), was found for the group of *Phoma* spp. from the marine environment. In contrast, HPLC-MS detection led to an average of 14.4 (SD 6.8) substances being detected in the terrestrial, and 13.2 (SD 6.5) in the marine fungal group, showing that there is no significant difference in the ability of each group to produce natural products.

The number of natural products appearing only once per strain during this investigation was also found to be very similar for both groups. In *Phoma* spp. of marine origin, 1.8 unique metabolites per strain occurred when detected by DAD, and 3.8 per strain when TSP-MS detection was used. Data obtained from terrestrial *Phoma* spp. showed 2.8 metabolites per strain to be unique by HPLC-DAD, and 3.6 if HPLC-MS was employed.

The accumulated data were examined statistically to see if the occurrence of (a) masses (derived from TSP-MS) in combination with retention times (R_t), and (b) UV maxima (DAD) in combination with R_t values, could be used as indicators for the assignment of the fungi to the marine (n = 26) or terrestrial (n = 16) group. In the first step, an exploratory data analysis was applied which aimed to prove the statistical significance of differences rather than to identify the discriminative potential masses and maxima. The underlying rationale for this approach was based on the fact that a variable (mass unit, UV maximum) is especially discriminative if it occurs in one group only. After having identified these variables in a second step it was proven that a linear combination of them can be used to predict a group affiliation as a 0/1 coded target variable.

(a) Mass units in combination with R_t

Of all masses detected (selection criterion: occurrence frequency ≥10 % per group), 11 appeared only in chromatograms of fungi of the terrestrial group [m/z/R_t (min) 151/36, 167/50, 181/58, 203/54, 205/53, 224/68, 243/42, 244/45, 245/46, 255/59, and 265/52], and 11 exclusively in the marine group (158/3, 167/41, 169/41, 175/4,

176/54, 185/37, 188/45, 193/35, 221/48, 221/59, 267/52). Three masses observed only in extracts of *Phoma* spp. of the marine group were defined as being redundant (251/49, 271/36, 272/47). The highest occurrence of a single mass in the marine group was 19 % (176/54, 221/48, 221/59) and in the terrestrial group 15 % (181/58, 243/42, 244/45). Thus, no exact (100 %) classification could be achieved by taking a single mass as a criterion for predictability. In order to calculate the predictability of a linear combination of the 22 mass data points, a multiple regression analysis was performed. The resulting coefficient with $R = 0.91$ $F(22,19) = 5.51$; $p < 0.00079$ exceeds the levels of significance ($p < 0.05$), which means 84 % (= R^2) of the variance of the target variable "group affiliation" can be explained when the 22 masses are known.

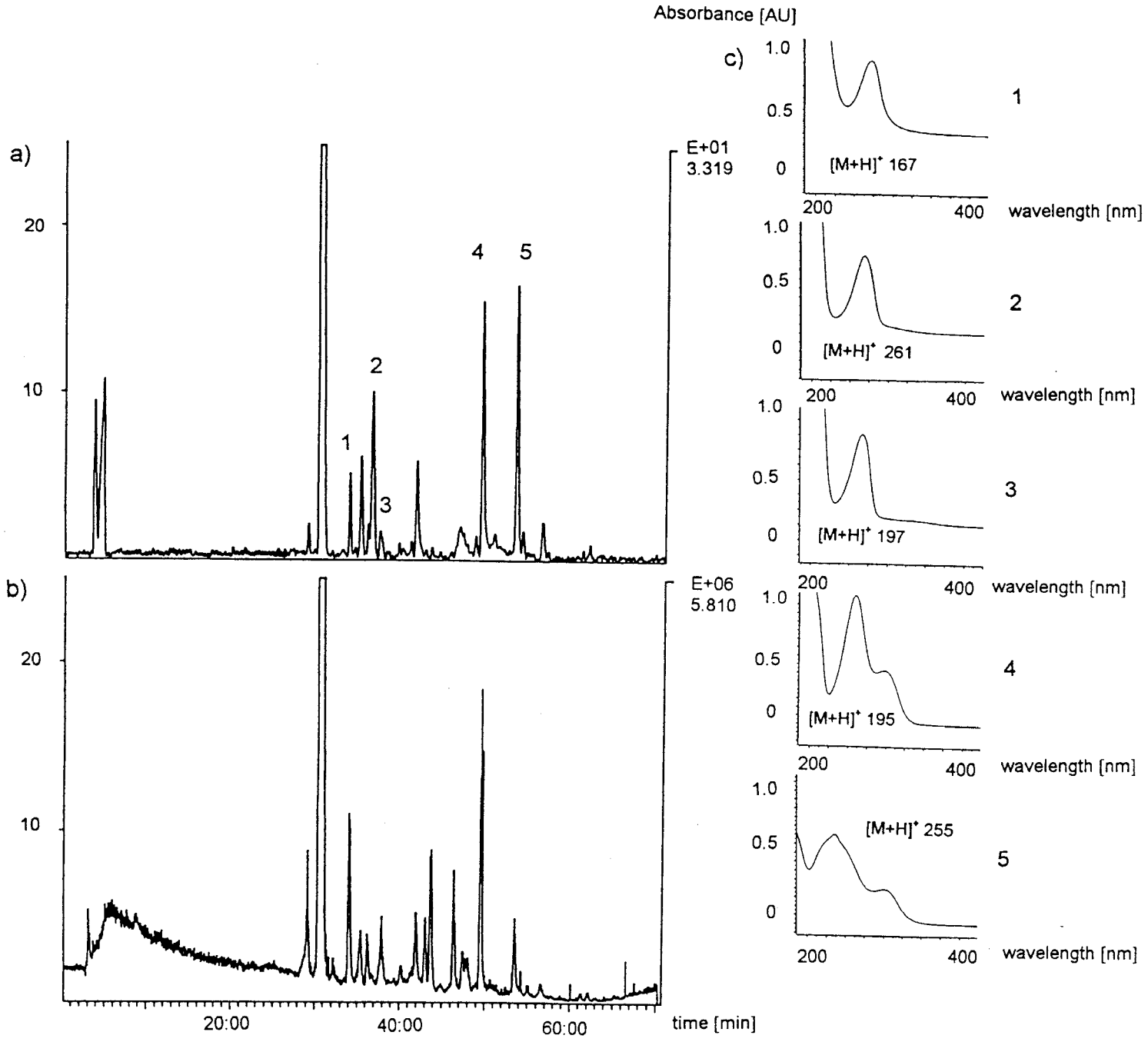


Figure 2. HPLC-chromatograms of an extract of the terrestrial *Phoma* species T7 with (a) UV-detection at 230 nm, and (b) TSP-MS detection. Panel (c) shows the UV spectra (in the range of 200-400 nm) and TSP-mass data [M+H$^+$] of five selected peaks (**1**-**5**).

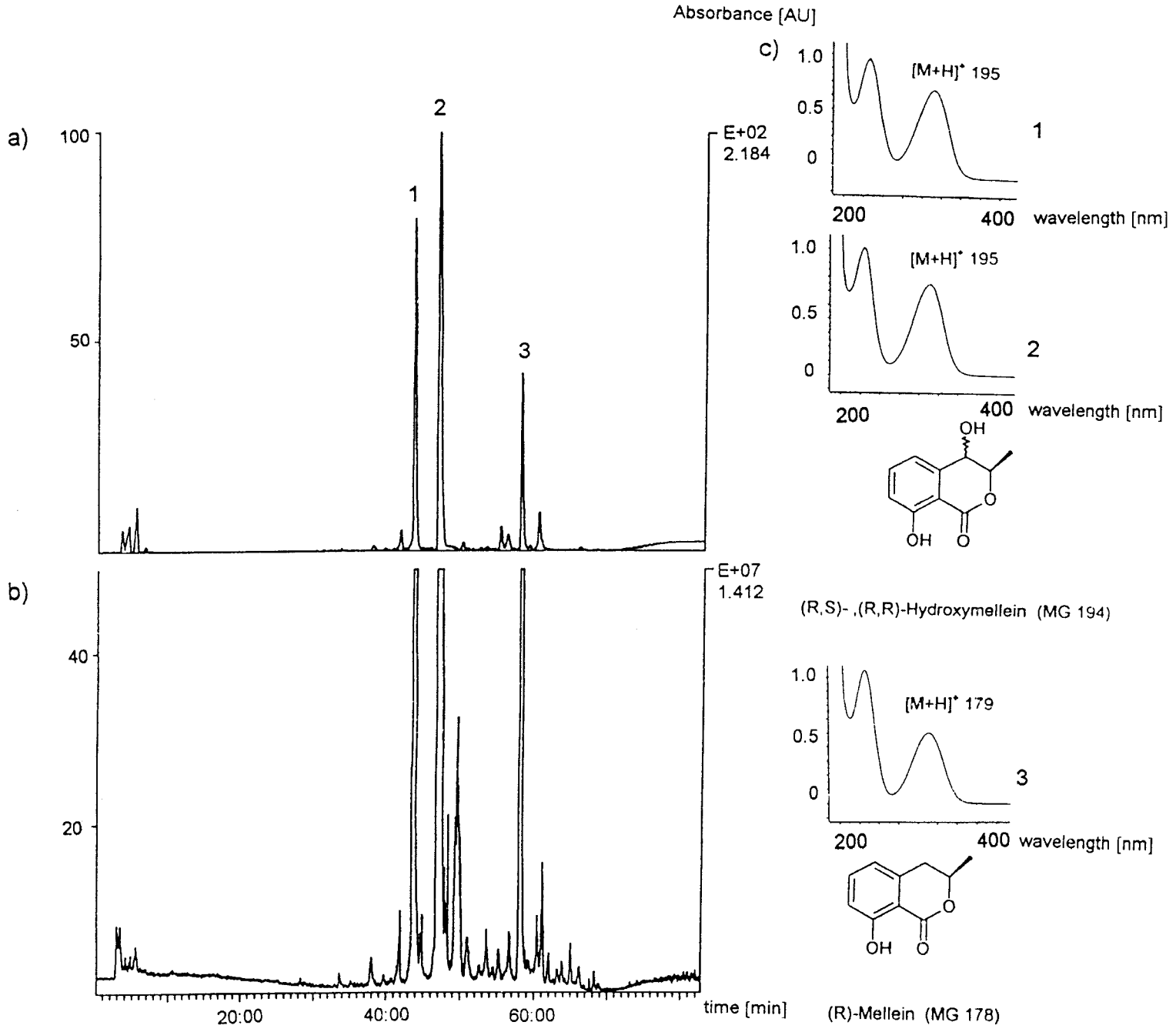


Figure 3. HPLC-chromatograms of an extract of the marine *Phoma* species M17 with (a) UV-detection at 230 nm, and (b) TSP-MS detection. Panel (c) shows the UV spectra (in the range 200-400 nm), the TSP-mass data [M+H$^+$] and suggested identities of three selected peaks (**1-3**).

(b) UV-maxima in combination with R_t

By using the same rationale as in (a), the UV maxima with discrimination potential were identified. Seven UV maxima [UV-maxima at R_t (min): 262/330 at 47, 220 at 32, 267/296 at 47, 245/311 at 43, 242/336 at 46, 286/212 at 43, and 227/271 at 56] appear only for metabolites from the terrestrial group, and six only for metabolites from the marine group (262 at 7, 282 at 10, 265 at 62, 278 at 36, 242 at 46, and 230/299 at 59). Multiple regression analysis with these data revealed that their linear combination has no statistical significant power for group prediction: $R = 0.68$ $F(13,28) = 1.92$; $p < 0.07$. Only 47 % of the target variables variance can be explained by the 13 chosen predictors.

For the graphical presentation of the results a hierarchical cluster analysis was performed with the amalgation algorithm “percent disagreement, weighted pair-group average”. In Figure 4 the resulting tree diagram of HPLC-TSP-MS data is shown. Separate clusters are formed by the fungi T1, T2, T3, T5 (from soil), and T4 and T6, the latter two having a remarkable distance from all the other fungi. The individuals T12 and T16 (from endophytic fungi), T10 and T15 (from soil/ endophytic fungi), and T14 and T9 are linked together on a high level of agreement. The fungi T7and T8 (from soil) were classified into a strong marine cluster M25, M24, M16, M15, and M13 (from sponges), and M2 (from an alga). Except for M20 (from a sponge), all other marine fungi were linked together with more than 85 % of agreement. The strains M6, M10, M17, and M23, all derived from sponges, and were classified into a strong cluster, as were M7, M12, M18 and M26, also originating from sponges. The *Phoma* spp. M3, M8 and M9, which were linked together on a level of more than 96 % agreement, were isolated from algae. The tree diagram (Fig. 4) is deeply sub-structured, which shows that neither the marine nor the terrestrial group is homogenous. Both groups clearly consist of different subgroups when the aforementioned mass/R_t for amalgation is applied. Figure 5 shows the tree diagram obtained from cluster analysis of the HPLC-DAD data. On the lowest level of the diagram one strong cluster is formed by fungi from both habitats (M1, M3, M6, M7, M8, M9, M10, M12, M15, M19, M23, M26, T14, T9, T8, T5, T3 and T2). On the next levels of linkage, three more terrestrial fungi are included (T1, T6 and T16) and four marine ones (M2, M4, M11 and M20). This means that on a level of only 15 %

disagreement nearly 60 % of investigated fungi (25 of 42) are clustered into one group (nine terrestrial and 16 marine ones). These results clearly show that the analysed UV maxima are inappropriate parameters for the detection of group affiliation for marine and terrestrial fungi belonging to the genus *Phoma*.

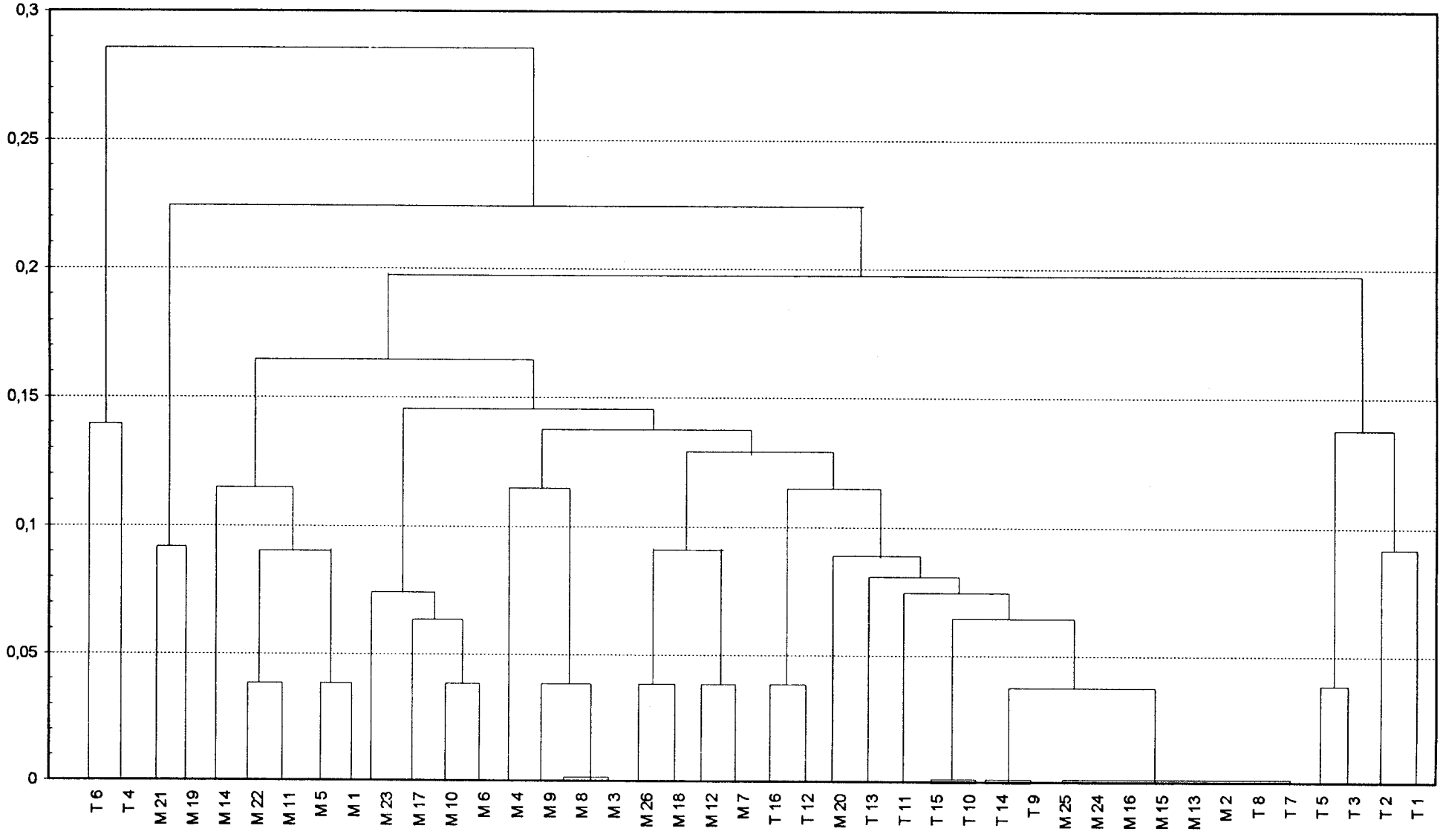


Figure 4. Tree-diagram resulting from cluster analysis of HPLC-MS data where the prefix T represents a terrestrial *Phoma* sp. and the prefix M a marine-derived *Phoma* sp.

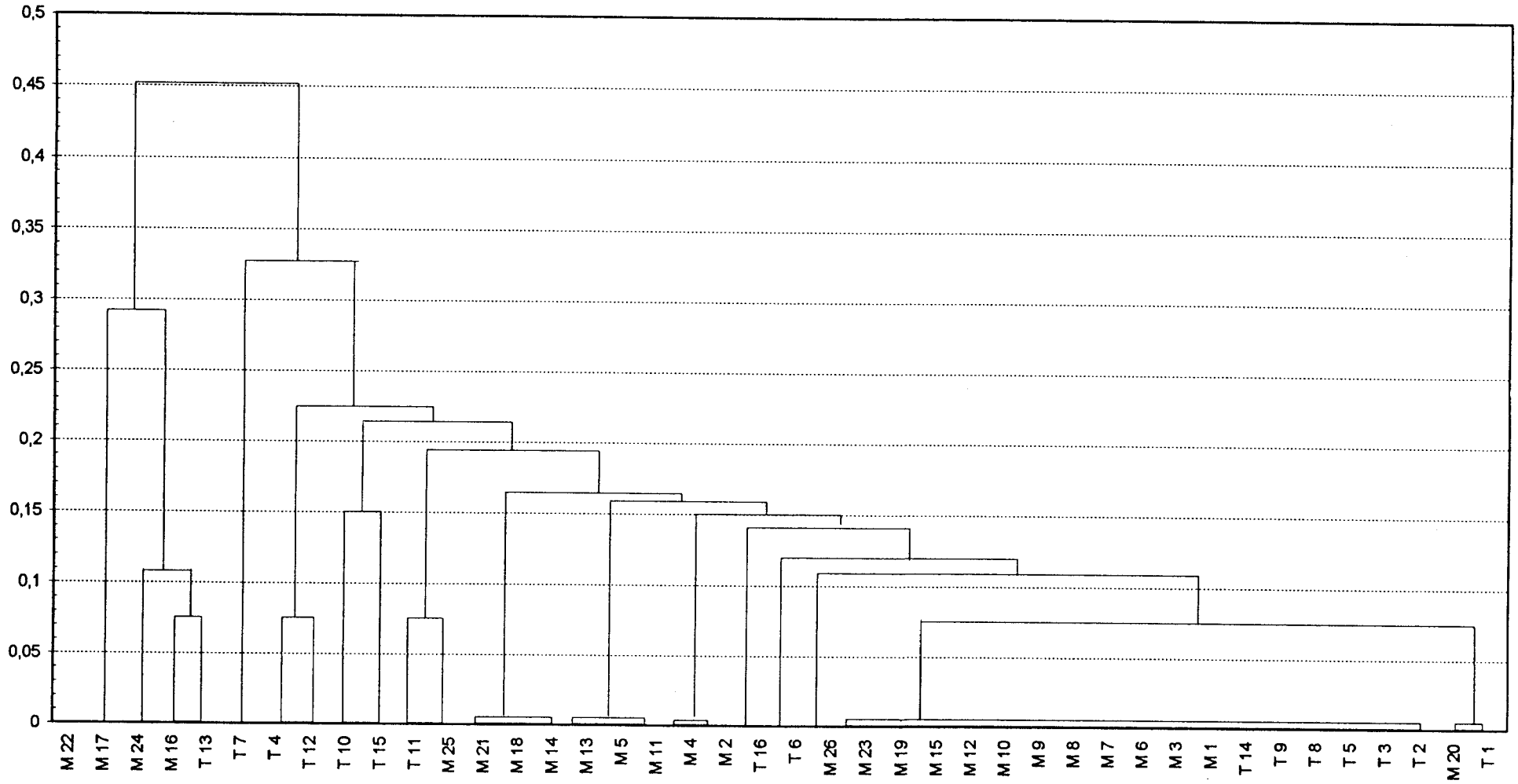


Figure 5. Tree-diagram resulting from cluster analysis of HPLC-DAD data where the prefix T represents a terrestrial *Phoma* sp. and the prefix M a marine-derived *Phoma* sp.

Clearly, all investigated *Phoma* spp., irrespective of their origin, produced many metabolites, as evidenced by the large numbers of peaks seen in the respective HPLC chromatograms. Interestingly, the average number of metabolites produced by a single strain, despite the high standard variation, and also the number of metabolites which occur exclusively in one sample, are almost the same in both groups. These similarities, regarding the biosynthetic capability of the investigated *Phoma* spp., are not surprising when their close taxonomic relationship is considered. As a great diversity of fungal strains were chosen, isolated from many different sources, e.g., algae, sponges, soil, endophytes of plants, similarities which could result from the choice of fungal strains, e.g., not diverse enough, can probably be excluded as a reason for the similar biosynthetic capabilities observed.

There are, however, clear differences between marine and terrestrial *Phoma* species. During HPLC-TSP-MS analysis especially, peaks were identified that were exclusively present in the chromatograms of the extracts from the fungi of marine origin, as were peaks in the chromatograms of the extracts from the fungi of terrestrial origin. Affiliation to the marine or terrestrial group could be achieved with an 84 % probability. The use of DAD data seems not to be specific enough for group classification as the probability of a correct prediction is less than 50 %. The fingerprint method of analysis of extracts has been shown before to be a useful method for the taxonomic classification of fungi (Laatsch *et al.*, 1993). It has to be considered, however, that the method of predictor selection employed in the present study followed a heuristic rationale which forces the hypothesis of predictability to be accepted. Therefore, any statistical results obtained have to be cross-validated by other studies and samplings.

The presented results indicate that many metabolites produced by marine-derived *Phoma* spp. are similar to those produced by their terrestrial counterparts. Both groups, however, seem to be distinguished by unique metabolic features. Their unique mass data in combination with retention times could be used as predictors for the affiliation of the extracts of *Phoma* spp. into terrestrial or marine origin which reveals that the metabolism of marine-derived *Phoma* spp. differs from that of terrestrial ones. This also indicates that marine-derived *Phoma* spp. (and perhaps

other marine fungi) are able to produce natural products not to be found in terrestrial *Phoma* species and, in a more general sense, that fungi derived from the sea might be regarded as valuable sources of new natural products.

4.3 Ascosalipyrrolone A, an antimicrobial alkaloid, from the obligate marine fungus *Ascochyta salicorniae*

Abstract

From the green alga *Ulva* sp., the endophytic and obligate marine fungus *Ascochyta salicorniae* was isolated. *A. salicorniae* was mass cultivated and found to produce the unprecedented and structurally unusual tetramic acid containing metabolites ascosalipyrrolones A (**1**) and B (**2**). Additionally, the new natural product ascosalipyrone (**3**) and the known metabolites **4** and **5** were obtained. Ascosalipyrrolone A (**1**) has antiplasmodial activity towards *Plasmodium falciparum* strains K1 and NF 54, as well as showing antimicrobial activity and inhibiting tyrosine kinase $p56^{lck}$.

Introduction

Terrestrial fungi are known as sources of secondary metabolites with significant therapeutic potential. The biosynthetic capabilities of marine fungi, however, are less well studied and interest in this group of organisms is increasing (König & Wright, 1996; Pietra, 1997). It was estimated by Kohlmeyer that one third of all known higher marine fungi are associated with algae (Kohlmeyer & Kohlmeyer, 1979). For this reason, and on the basis of the idea that algicolous fungi may produce metabolites as a means of dealing with their host plant, algae were regarded as a valuable source for the isolation of unusual and obligate marine fungal strains. In the last five years compounds with novel chemical structures and biological activities have been isolated from algicolous fungi, e.g., communesins (Numata *et al.*, 1993), leptosins (Takahashi *et al.*, 1994a and b, 1995a), penochalasins (Numata *et al.*, 1996), penostatins (Takahashi *et al.*, 1996; Iwamoto *et al.*, 1998), and pyrenocine E Amagata *et al.*, 1998d). All of these compounds are cytotoxic towards cultured P-388 lymphocytic leukemia cells. Further examples of biologically active compounds isolated from fungi associated with algae include the halymecins with antimicroalgal activity (Chen *et al.*, 1996), exumolides A and B (Jenkins *et al.*, 1998a), also with antimicroalgal activity and finally some sesquiterpenoid nitrobenzoyl esters which were shown to be cytotoxic towards HCT-116 human colon carcinoma cells (Belofsky *et al.*, 1998). Terrestrial species of the genus *Ascochyta* have been studied for their natural product content, with pinolidoxin being isolated from *A. pinodes* (Evidente *et al.*, 1993), chrysanthones B and C from *A. chrysanthemi* (Arnone *et al.*, 1990), and ascochalasin from *A. heteromorpha* (Capasso *et al.*, 1988), indicating the genus *Ascochyta* to have a highly developed and diverse secondary metabolism.

During our investigations dealing with the isolation, cultivation and screening of fungal strains associated with marine algae, the obligate marine fungus *Ascochyta salicorniae* was obtained from the marine green alga *Ulva* sp., collected from the North Sea, Tönning, Germany. Algal material was surface sterilized with EtOH 70 % to ensure fungi were only isolated from the inner algal tissue. Thus, *A. salicorniae* is assumed to be an algal endophyte. The EtOAc extract of this fungal isolate was found to have antimicrobial activity. On the basis of this activity the fungus was further investigated in order to identify the biologically active natural products.

Materials and methods

Isolation and Taxonomy of the fungus

Algal material was collected from the North Sea, Tönning, Germany. After sterilization with 70 % EtOH algal samples were rinsed with sterile sea water and pressed onto agar plates to detect any residual fungal spores on the surface of the algae. Sterilized algae were then cut into small pieces and placed on agar plates containing isolation medium: 30 g/L blended *Ulva* sp., 15 g/L agar, and 1000 mL sea water from the sample collecting site. After autoclaving the antibiotics benzylpenicillin and streptomycin sulphate were added by sterile filtration. Fungal colonies growing out of the algal tissue were transferred to medium for sporulation (1.0 g glucose, 0.1 g yeast extract, 0.5 g peptone from meat, enzymatic digest, 15 g agar, and 1000 mL sea water, pH 8) in order to enable taxonomy of the isolates.

Cultivation

The microorganism was cultured at 20 °C for 40 days in eight litres of solid medium containing 20 g/L biomalt extract, 8 g/L agar and 80 % ASW.

Biological activity, see chapter 3.5.

Extraction and Isolation

Fungal mycelia were separated mechanically from the culture agar and extracted first with MeOH (6.5 L), and then with EtOAc (6.5 L) after being blended with an Ultra Turrax model T25 at 8000 min^{-1}. Prior to extraction with 24 L EtOAc followed by 24 L of n-butanol the solid medium was diluted with H_2O and mixed using the Ultra Turrax T 25. The resultant EtOAc extract (11.4 g) was purified employing a combination of chromatographic techniques. First, it was passed over normal phase silica (vacuum liquid chromatography, VLC) using a gradient starting with CH_2Cl_2 then EtOAc and finally MeOH as eluent to yield 17 fractions each of 250 mL. VLC fraction 4 (85 mg, eluted with EtOAc/CH_2Cl_2 1:9) in which the antimicrobial activity was concentrated was subjected to further normal phase VLC employing EtOAc:MeOH 95:5 as eluent to give 115 fractions. Fractions 4-6 were combined after TLC examination (32 mg),

and yielded compounds **1** (8.7 mg, 1.1 mg/L) and **2** (0.8 mg, 0.1 mg/L) after purification by RP-18 HPLC (Spherisorb ODS S2 5µm, 8 mm×25 cm) using acetonitrile:water 85:15 as eluent. VLC fraction 8 from the first separation was further investigated on the basis of its ^{1}H-NMR spectrum which contained a number of interesting resonances in the δ 4.0 to 10.4 range. Separation of this fraction by VLC, using normal phase silica and a gradient starting with CH_2Cl_2 then EtOAc and finally MeOH as eluent give 13 fractions. Fractions 4 and 5 were combined after TLC examination and further separated by RP-18 HPLC using acetonitrile:water 60:40 as eluent to yield compound **3** (19 mg, 2.4 mg/L). Compounds **4** (2 mg, 0.25 mg/L), and **5** (11 mg, 1.4 mg/L) were obtained by normal phase HPLC separation of fraction 3 from VLC fraction 8 using hexane/acetone 70:30 as eluent.

Results and Discussion

In the present study the isolation, structure elucidation and biological activities of five secondary metabolites obtained from *Ascochyta salicorniae* are described. The fungus was cultivated on a solid biomalt medium with added artificial sea salt. Successive fractionation of the EtOAc extract by vacuum-liquid chromatography (VLC), and normal (Si-60) and reversed (RP-18) phase HPLC yielded compounds **1**-**5**. Compounds **1** and **2** are unusually substituted tetramic acid derivates and most interesting as a result of their antimicrobial activity.

Ascosalipyrrolone A (**1**) has the molecular formula $C_{27}H_{41}NO_3$ as deduced by accurate mass measurement. Its ^{13}C NMR spectrum contained 27 signals (see Table 9). From the ^{13}C NMR spectroscopic data (^{1}H decoupled and DEPT) it was evident that five of the eight elements of unsaturation, indicated by the molecular formula of **1**, could be attributed to three carbon-carbon double bonds [δ 155.8 (d, C-20), 137.0 (s, C-19), 132.4 (s, C-2), 128.6 (d, C-1), 135.2 (s, C-14), 122.7 (d, C-15)], and two carbonyl groups [δ 201.9 (s, C-18), 167.5 (s, C-23)], these being the only multiple bonds within **1**; the molecule is thus tricyclic. The ^{1}H and ^{13}C NMR spectra further revealed the presence of five methylene groups [δ 19.2 (t, C-26), 32.0 (t, C-25), 38.5 (t, C-7), 39.0 (t, C-9)], one of which was attached to oxygen [δ 63.2 (t, C-24)], six methine groups [δ 32.9 (d, C-8), 37.0 (d, C-6), 39.4 (d, C-10), 41.6 (d, C-5),

43.2 (d, C-4), 57.5 (d, C-3)], two of them adjacent to methyl groups [δ 0.67 (d, H_3-12); 0.91 (d, H_3-11)], five methyl groups [δ 1.57 (s, H_3-28); 1.42 (brs, H_3-13); 0.89 (t, H_3-27); 1.42 (brs, H_3-16); 1.47 (brs, H_3-17)], and a quaternary carbon bound to oxygen and nitrogen [δ 87.1 (s, C-21)].

From the ^{1}H-^{1}H-COSY spectrum three fragments of **1** could be deduced (see Fig. 6). Thus, ^{1}H-^{1}H couplings were observed between H_3-12 and H-6, between H-6 and H_2-7, between H_2-7 and H-8, between H-8 and H_2-9, between H_2-9 and H-10, between H-10 and H-1, between H-10 and H-5, between H-5 and H-4, between H-4 and H-3 and between H-8 and H_3-11. Cross-peaks between the resonances for H-15 and H_3-16 indicated these protons also to couple. Additionally, ^{1}H-^{1}H couplings were seen between the proton bound to nitrogen (H-22) and H-20 in the pyrrolone and between H_2-24 and H_2-25, H_2-25 and H_2-26, and H_2-26 and H_2-27, indicating the presence of a butoxyl function. This information together with data obtained from the ^{1}H-^{13}C HMBC spectrum allowed the planar structure of **1** to be deduced.

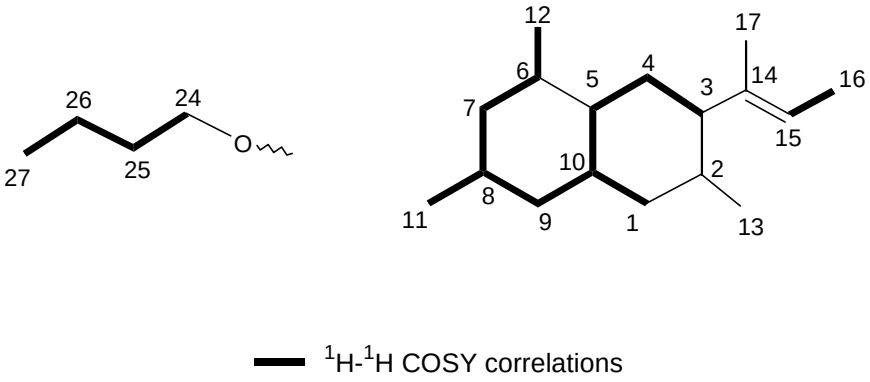


Figure 6. Selected partial structures of ascosalipyrrolone A (**1**) deduced from ^{1}H-^{1}H COSY correlations.

Thus, diagnostic HMBC correlations from the resonance for H_3-12 to those for C-5, C-6 and C-7 permitted the Decalin ring to be completed. Further proof of this system came from HMBC correlations between the resonance for H_3-13 and those of C-1, C-2 and C-3 clearly positioning CH_3-13 at C-2. HMBC correlations between the resonance for H_3-17 and those for C-3, C-14 and C-15 showed the 2-butenyl moiety to be placed at C-3. Further, ^{1}H-^{13}C HMBC correlations observed between the resonances for H-20 and the resonances for C-18, C-19, C-21, C-23, and C-28, and

from the resonance for the NH proton (N-22) to those for all carbon atoms of the pyrrolone ring system, i.e. C-19, C-20, C-21, C-23 supported the existence of this moiety, as did the HMBC correlations between the resonance for H_3-28 and those for C-19, C-20, C-21 and C-24, thus completing the third ring within **1**. The linkage between the butoxyl group and C-21 was evident from the ^{1}H-^{13}C HMBC correlation between the resonances for H_2-24 and that for C-21. The remaining connectivities between C-4, C-18 and C-19 followed by deduction.

The ^{1}H-NMR resonance for H-22 (NH) of the pyrrolone moiety was very broad. This can be explained due to tautomerism as depicted in Fig.7. The ^{1}H NMR spectrum contained resonances for both tautomers.

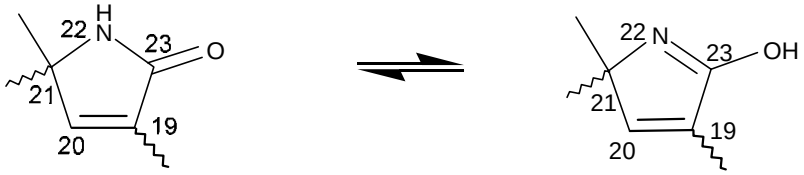
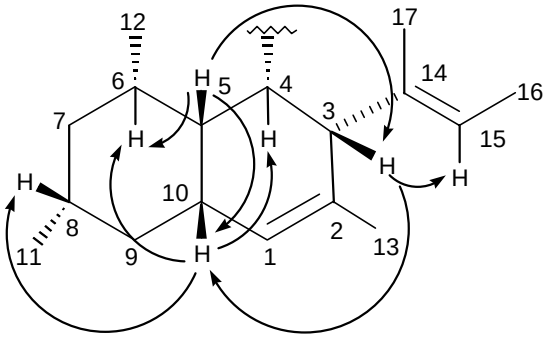


Figure 7. Tautomeric forms of the pyrrolone moiety of **1**.

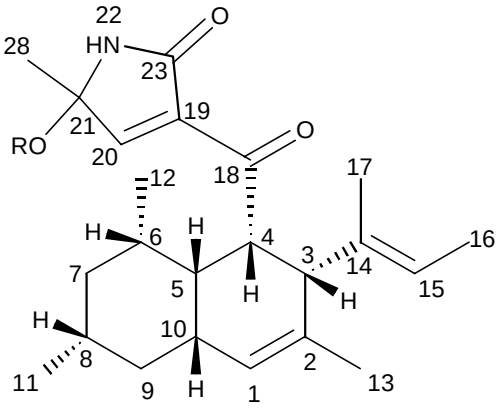
The relative stereochemistry of **1** was determined from the results of NOE difference measurements (Fig. 8). Thus, irradiation at the resonance frequency of H-5 caused enhancement of the resonance for H-10, and *vice versa*, and of the resonance for H-3, indicating the Decalin to be cis-fused and H-3 also to be on the same side of the molecule, β-oriented. Enhancement of the resonance for H-20 was caused by irradiation at the resonance frequency of H-15, indicating both these moieties to be on the same side of **1**. Irradiation at the resonance frequency of H-3 caused enhancement of the resonance for H-15 and *vice versa*, revealing the *E*-configuration of the $\Delta^{14,15}$ double bond. Irradiation at the resonance frequency of H-10 caused enhancement of the resonance for H-6 showing them also to be on the same side of the molecule. Finally, H_3-11 was deduced as having an α-orientation on the basis of a NOE interaction between H-8 and H-10. The configuration at C-21 remained unassigned as it could not be related in a relative sense to the other chiral centers in the molecule. Thus, the relative configuration of ascosalipyrrolone A (**1**) is 3*R**, 4*S**, 5*S**, 6*S**, 8*R**, 10*R**.



Arrows show NOEs between both involved protons

Figure 8. Diagnostic NOEs for ascosalipyrrolone A (**1**).

Compound **2** was purified in the same way as **1**. The small amount of **2** isolated precluded ^{13}C and 2D NMR spectral measurements. Comparison of its ^{1}H-NMR data with those of compound **1** (Table 9), however, indicated the only difference between the two data sets to be the absence of the resonances for H_2-24, H_2-25, H_2-26 and H_3-27, the O-butyl moiety, in **2**, and the presence of a resonance for a methoxyl group instead (δ 3.1, 3H, s). This deduction was also supported by EIMS, IR and UV measurements.



1 R= -$CH_2CH_2CH_2CH_3$ (24 25 26 27)

2 R= -CH_3 (24)

Table 9. ^{1}H (600 MHz, $CDCl_3$) and ^{13}C (150 MHz, $CDCl_3$) NMR data for ascosalipyrrolone A (**1**) and ^{1}H NMR data (200 MHz, $CDCl_3$) for ascosalipyrrolone B (**2**).

position	δ C of **1**	type[a]	δ H of **1**	HMBC[b] of **1**	NOE[c] of **1**	δ H of **2**
1	128.6	CH	5.58 (d, *J* = 6.1 Hz)	3,5,10,13	9, 10	5.57 (d)
2	132.4	C	-	-	-	-
3	57.5	CH	2.87 (brd, *J* = 7.6 Hz)	1,2,14,15	15	2.86 (d)
4	43.2	CH	4.07 (brm)	-	1, 7, 20	4.08 (brm)
5	41.6	CH	2.32 (m)	-	3, 6, 10	2.31 (brm)
6	37.0	CH	1.73 (m)	-	-	1.69
7	38.5	CH_2	1.17 (m)	-	-	1.19
			1.29 (m)			1.28
8	32.9	CH	1.44 (m)	-	-	1.44 (m)
9	39.0	CH_2	1.65 (brd, *J*=13.0 Hz)	-	-	1.55
			0.95 (ddd, *J*=13.0 Hz)			0.91
10	39.4	CH	2.07 (brm)	1,2,4,9	4, 5, 6, 9	2.08 (m)
11	22.4	CH_3	0.91 (d, *J*=6.6 Hz)	2,8; 9	-	0.92 (d)
12	23.5	CH_3	0.67 (d, *J*=7.4 Hz)	5,6,7	-	0.68 (d)
13	21.0	CH_3	1.42 (brs)	-	-	1.41 (brs)
14	135.2	C	-	-	-	-
15	122.7	CH	5.12 (m)	1,3,16	3, 20	5.11 (m)
16	13.2	CH_3	1.42 (brs)	-	-	1.43 (brs)
17	11.9	CH_3	1.47 (brs)	-	-	1.46 (brs)
18	201.9	C	-	-	-	-
19	137.0	C	-	-	-	-
20	155.8	CH	7.38 (m)	18, 19, 21, 23, 28	4, 24	7.32 (m)
21	87.1	C	-	-	-	-
23	167.5	C	-	-	-	-
24	63.2	CH_2	3.33 (m)	21, 25, 26	-	3.09 (s)
			3.07 (m)			($-OCH_3$)
25	32.0	CH_2	1.46 (m)	-	-	-
26	19.2	CH_2	1.32 (m)	-	-	-
27	13.8	CH_3	0.89 (t, *J* = 7.3 Hz)	25, 26	-	-
28	25.1	CH_3	1.57 (s)	-	20, 24	1.58 (s)
22 (NH)	-	-	5.91 (brs)	19, 20, 21, 23	20, 24, 28	5.65 (brs)

[a] Attached protons as determined by DEPT (s = C, d = CH, t = CH_2, q = CH_3).

[b] Numbers represent carbon atoms that are observed to long-range CH couple with the proton(s) associated with this data row.

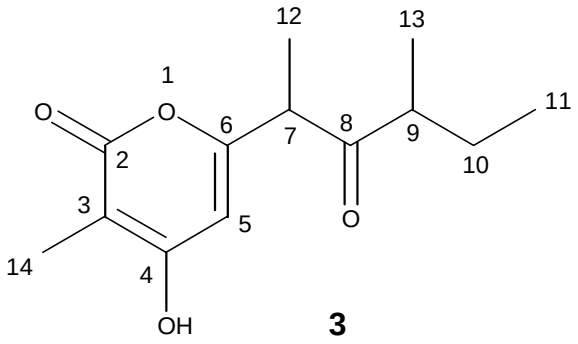
[c] Enhanced proton signals as observed by difference NOE measurements.

Ascosalipyrone (**3**) was found to have the molecular formula $C_{13}H_{18}O_4$ by MS. Its ^1H and ^{13}C NMR spectra contained resonances (Table 10) attributable to four methyl groups [δ 0.82 (t, H_3-11), 1.05 (d, H_3-12), 1.38 (d, H_3-13), 1.94 (s, H_3-14)], one methylene group [δ 26.1 (t, C-10)], three methine groups [δ 101.6 (d, C-5), 49.3 (d, C-7), 47.1 (d, C-9)], a quaternary olefinic carbon [δ 99.8 (s, C-3)], two oxygenated quaternary olefinic carbons [δ 160.7 (s, C-6, lactone), 166.2 (s, C-4, hydroxyl)], and two carbonyls [δ 167.6 (s, C-2, lactone), 211.5 (s, C-8, ketone)]. The pyrone ring of ascosalipyrone was established as follows. Diagnostic long-range ^1H-^{13}C heteronuclear couplings observed from the resonance for H_3-14 to the resonances for C-2, C-3 and C-4 revealed H_3-14 to be connected to C-3, which is further bonded to the lactone carbon C-2 and to C-4. Further HMBC correlations seen between the resonance for H-5 and those for C-3, C-4, and C-6 revealed C-5 to be bonded to C-4 and C-6, thus completing the 4-hydroxy-2-pyrone ring system.

The ^1H-^1H COSY spectrum of **3** contained cross-peaks between the resonance for H_3-11 and those for H_2-10, between the resonances for H_2-10 and those of H-9, and those for H-9 and for H_3-13 showing C-11 to be bonded to C-10, C-10 to C-9, and C-9 to C-13 generating an iso-butyl moiety. Additionally, a ^1H-^1H COSY cross-peak between the resonances for H_3-12 and H-7 gave the C-7, C-12 ethyl moiety. Further analysis of the HMBC spectrum showed correlations from the resonances for H-7, H-9, H_2-10, H_3-12 and H_3-13 to C-8 indicated the C-8 carbonyl group to bond to both C-7 and C-9. As the resonance for H-7 showed HMBC correlations to those for C-6 and C-5, C-7 must bond to C-6 thus completing the planar structure of **3**. The proposed structure of **3** was supported by the results of NOE measurements. Thus, irradiation at the resonance frequency of H-5 caused enhancement of the resonances of H-7, H-9 and H_3-12, supporting the positioning of H-5. Therefore, compound **3** was unambiguously assigned as 6-(1,3-dimethyl-2-oxopentyl)-4-hydroxy-3-methyl-2*H*-pyran-2-one.

Compound **3** was most probably isolated as a mixture of diastereomers. This is evident from its ^{13}C NMR spectrum which contains double resonances for C-6 (δ 160.7 and 160.5), C-7 (δ 49.3 and 49.1), C-8 (δ 211.5 and 211.3), C-9 (δ 47.1 and 46.9), C-10 (δ 26.1 and 25.7), C-12 (δ 14.5 and 14.4) and C-13 (δ 16.4 and 15.9).

Attempts to separate the isomers by GC-MS proved unsuccessful. The structure of **3** resembles that of phomapyrone B which was isolated from the phytopathogenic fungus *Leptosphaeria maculans*, the asexual stage of *Phoma lingam* (Pedras *et al.*, 1994). The only difference between the two molecules being the presence of the methyl group at C-9 in the side chain of **3**.



3

Table 10. ^{1}H (300 MHz, $CDCl_3$) and ^{13}C (75 MHz, $CDCl_3$) NMR data for ascosalipyrone (**3**).

position	δ C	type[a]	δ H	HMBC[b]	NOE[c]
2	167.6	C	-	-	-
3	99.8	C	-	-	-
4	166.2	C	-	-	-
5	101.6	CH	6.21 (s)	3,4,6,7,8,14	7,9,12,13,OH
6	160.7	C	-	-	-
7	49.3	CH	3.77 (q, *J* = 7.0 Hz)	5,6,8,12	-
8	211.5	C	-	-	-
9	47.1	CH	2.68 (m)	8,10,11,13	-
10	26.1	CH_2	1.69 (m)	8,9,11,13	-
			1.38 (m)		
11	11.6	CH_3	0.82 (t, *J* = 7.3 Hz)	9,10	-
12	14.4	CH_3	1.05 (d, *J* = 7.0 Hz)	6,7,8	-
13	15.9	CH_3	1.38 (d, *J* = 7.2 Hz)	8,9,10	-
14	8.2	CH_3	1.94 (s)	2,3,4	-
OH	-	-	9.7 (brs)	-	-

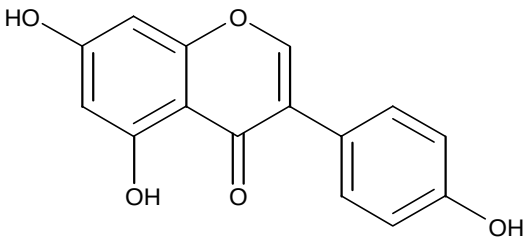
[a] Attached protons as determined by DEPT (s = C, d = CH, t = CH_2, q = CH_3).

[b] Numbers represent carbon atoms which are observed to long-range CH couple with the proton(s) associated with this data row.

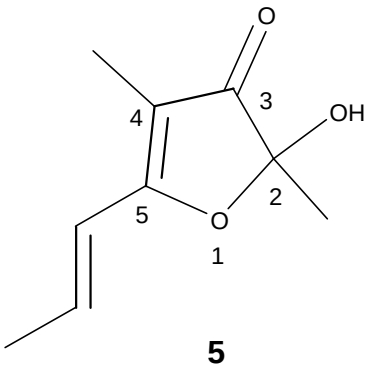
[c] Enhanced proton signals as observed by difference NOE measurements.

Compound **4** was identified as genistein by comparison of its spectroscopic data with published values (Aida *et al.*, 1995). Isoflavone compounds such as **4** are known to occur commonly in higher plants, but they have also been isolated from bacteria (Hudson & Bentley, 1969; Ganguly & Sarre, 1970; Hazato, 1979; Ogawara, 1986), and from a culture of the fungus *Aspergillus niger* (Umezawa *et al.*, 1975). In most cases, as in this study, the fermentation media contain plant based nutrients, and so it is not clear if **4** was produced by the microorganism or was isolated as an original component of the medium. On several other occasions during our studies the same cultivation medium was used and genistein was never found. It can, however, be speculated that genistein may be present in the medium in a glycosidic form that is normally not extracted with EtOAc and that some fungi, e.g., *Ascochyta salicorniae*, are able to enzymatically hydrolyze the glycosidic bonds. This is a possible explanation as to why we have never found this compound before. Genistein was reported to inhibit the enzymes *β*-galactosidase (Hazato *et al.*, 1979), dopa decarboxylase, histidine decarboxylase, and catechol-O-methyltransferase (Umezawa *et al.*, 1975).

Compound **5**, 2,3-dihydro-2-hydroxy-2,4-dimethyl-5-trans-propenylfuran-3-one, was identified by comparison of its ^{1}H and ^{13}C NMR data with published values. This compound was first isolated from culture filtrates of the phytopathogenic fungus *Stemphylium radicinum* (Grove, 1971).



4



5

Compounds **1** and **2** are deoxy tetramic acids and as such belong to an extremely rare group of natural products. Two compounds similar to **1** and **2** have been reported from the fermentation broth of other fungi. Oteromycin, an antagonist of the ET_B receptor, was obtained from two strains of an unidentified fungus (Singh *et al.*, 1995), and ZG-1494α, an inhibitor of platelet-activating factor (PAF) acetyltransferase was isolated from *Penicillium rubrum* (West *et al.*, 1996). Significant structural differences between ascosalipyrrolones A and B (**1** and **2**), and oteromycin and ZG-1494α are the substitution and stereochemistry of the Decalin ring system and the presence of a methyl group in **1** and **2** at C-21, instead of the phenyl or 4-hydroxyphenyl moiety found in oteromycin and ZG-1494α, respectively. The finding of the new and structurally unusual compounds reported here from *A. salicorniae* further supports the proposition that marine-derived fungi are an extremely interesting and valuable source of novel natural products.

The antimicrobial, antialgal, nematicidal, antiplasmodial, antitrypanosomal and cytotoxic properties as well as brine shrimp lethality of all compounds, except **2**, were assessed. The HIV-1 reverse transcriptase and tyrosine kinase ($p56^{lck}$) inhibitory activities of these compounds were also investigated using ELISA (Table 11). Ascosalipyrrolone A (**1**) was found to exhibit antimicrobial activity in agar diffusion assays against *Bacillus megaterium* (5 mm), *Mycotypha microsporum* (4 mm) and *Microbotryum violaceum* (2 mm) at a concentration of 50 μg/filter disk. It also inhibited the enzyme tyrosine kinase to 70 % of its activity at a concentration of 40 μg/mL and to 23 % at a concentration of 200 μg/mL. In bio-assays for antiplasmodial activity compound **1** was found to inhibit the growth of two strains of *Plasmodium falciparum* at levels consistent with it being considered as a moderate antiplasmodial agent (see Table 11). Compound **1** exhibited significant activity against *Trypanosoma cruzi* and also against *Trypanosoma brucei* subsp. *rhodesiense*, as well as having cytotoxic activity against rat skeletal muscle myoblast cells and mouse peritoneal macrophages (Table 12). In the applied test systems ascosalipyrone (**3**) demonstrated no activities. The two known compounds, genistein (**4**) and 2,3-dihydro-2-hydroxy-2,4-dimethyl-5-trans-propenylfuran-3-one (**5**), we found to inhibit the enzyme tyrosine kinase, an activity already reported for genistein (Umezawa, 1994; Ogawara *et al.*, 1986). Additionally, compound **5** exhibited activity

against *Tr*. *brucei* subsp. *rhodesiense* and *Tr. cruzi* and also weak antifungal activity against *Microbotryum violaceum* and *Eurotium repens*. In assays with *Artemia salina* (brine shrimp) and *Caenorrhabditis elegans* (nematode), none of the tested compounds had any effects.

Table 11. Antibacterial, antifungal, enzyme inhibitory (tyrosine kinase) and antiplasmodial activities of compounds **1**, **4**, **5**.

	Antimicrobial activity		Tyrosine Kinase^{lck} activity		Antiplasmodial activity (IC_{50})	
Compound	Bacteria[a]	Fungi[b]			K1 [d]	NF54[d]
	[50 μg/disc]	[50 μg/disc]	[200 μg/mL]	[40 μg/mL]	[ng/mL]	[ng/mL]
1	Bm 5 mm ti	Mv 2 mm ti; Mm 4 mm ti	23[c]	70[c]	736	378
4	na	na	7[c]	55[c]	9272	6054
5	na	Mv 1 mm gi; Er 2 mm gi	9[c]	56[c]	1763	1760
Chloroquine	-	-	-	-	97	4

na = not active; ti = total inhibition zone in mm, no colonies of the test organism were growing within the inhibition zone; gi = growth inhibition zone in mm, some colonies of the test organism were growing within the inhibition zone. Inhibition zones were measured from the edge of the filter discs.

[a] Tested against *Bacillus megaterium* (Bm) and *Escherichia coli* (Ec), benzylpenicillin and streptomycin sulphate were positive controls: inhibition zones of benzylpenicillin 2 mm (Ec), 17 mm (Bm); inhibition zones of streptomycin sulphate 2 mm (Ec), 10 mm (Bm). Test concentration: 50 μg/disc.

[b] Tested against *Eurotium repens* (Er), *Fusarium oxysporum* (Fo), *Microbotryum violacea* (Mv), and *Mycotypha microspora* (Mm), miconazol was positive control: inhibition zone 25 mm (Mv), 25 mm (Er), 3 mm (Fo), and 7 mm (Mm), respectively. Test concentration: 50 μg/disc.

[c] Values give % residual tyrosine kinase (TK p56^{lck}) activity. Lck = lymphocytic kinase.

[d] Antiplasmodial activity was measured against two reference strains of *Plasmodium falciparum* K1 (Thailand; resistant to chloroquine and pyrimethamine), and NF 54 (an airport strain of unknown origin; susceptible to standard antimalarials).

Table 12. Activities against *Trypanosoma brucei* subsp. *rhodesiense*, *Trypanosoma cruzi* and cytotoxicity of compounds **1** and **5**.

	T.b. rhodesiense	*T. cruzi*	Cytotoxicity (MIC)	
Compound	MIC [µg/mL]	MIC [µg/mL]	L-6[a] [µg/mL]	Macrophages[b] IC_{50} [µg/mL]
1	30	1.1	3.7	2.2
5	90	30	10	-
Melarsoprol[c]	0.072	-	-	-
Benznidazole[c]	-	30	-	-

[a] Rat skeletal muscle myoblast cells.

[b] Mouse peritoneal macrophages.

[c] Positive controls.

Ascosalipyrollone A (**1**) was obtained as colourless amorphous powder; $[\alpha]^{20}_D$ = -51.3° (*c* = 0.16, EtOH); UV (EtOH) λ_{max} (log ε) 211 (4.1), 225 sh (3.89), 267 (3.08); IR (film) ν_{max} 3300, 1710, 1630 cm^{-1}; ^1H and ^{13}C NMR data see Table 9; EIMS *m/z* 427 [M^+] (88), 409 (26), 392 (44), 354 (72), 239 (54), 231 (60), 201 (88), 175 (44), 159 (72), 135 (54), 124 (100), 105 (92), 91 (66), 69 (58), 55 (80); HREIMS *m/z* 427.309 (calcd. for $C_{27}H_{41}NO_3$ 427.309).

Ascosalipyrollone B (**2**) was isolated as colourless powder; $[\alpha]^{20}_D$ = 0.0° (*c* = 0.05, EtOH); UV (EtOH) λ_{max} (log ε) 207 (4.38), 225 (4.08), 273 (3.24); IR (film) ν_{max} 3580, 1725, 1605 cm^{-1}; ^1H NMR data see Table 9; EIMS *m/z* 385 [M^+], 352 (40), 336 (32), 293 (36), 239 (38), 231 (45), 201 (52), 175 (28), 159 (36), 149 (100), 124 (46), 109 (42), 71 (44), 57 (58); HREIMS *m/z* 385.261 (calcd. for $C_{24}H_{35}NO_3$ 385.261).

Ascosalipyrone (**3**) was obtained as yellow amorphous powder; $[\alpha]^{20}_D$ = -61.3° (*c* = 0.32, EtOH); UV (EtOH) λ_{max} (log ε) 210 (3.81), 290 (3.51); IR (film) ν_{max} 2965, 1715, 1630 cm^{-1}. ^1H and ^{13}C NMR data see Table 10; EIMS 238 [M^+] (18), 154 (100), 85 (18), 57 (54); HREIMS *m/z* 238.120 (calcd. for $C_{13}H_{18}O_4$ 238.121).

Genistein (**4**): UV (MeOH) λ_{max} (log ε) 262 (3.69), 209 (4.0); Lit. (Ganguly & Sarre, 1970) 260 ε (38460); IR (film) 3400, 2955, 2925, 2850, 2360, 2340, 1730, 1715 cm^{-1}; ^{1}H NMR ppm 13.03 (s), 8.53br (s), 8.17 (s), 7.45 (d, *J* = 8.4 Hz), 6.90 (d, *J* = 8.4 Hz), 6.42 (d, *J* = 2.2 Hz), 6.28 (d, *J* = 2.2 Hz); Lit. (Ganguly & Sarre, 1970) ppm 12.96 (s), 8.3 (s), 7.45 (d, *J* = 10 Hz), 6.8 (d, *J* = 10 Hz), 6.4 (d, *J* = 2 Hz), 6. 2 (d, *J* = 2 Hz). ^{13}C NMR ppm 181.7 (s), 165.0 (s), 163.9 (s), 159.1 (s), 158.5 (s), 154.3 (d), 131.2 (d), 131.2 (d), 124.0 (s), 123.0 (s), 116.0 (d), 116.0 (d), 106.2 (s), 99.8 (d), 94.5 (d). ^{13}C NMR (Aida *et al.*, 1995) ppm 181.7 (s), 165.0 (s), 163.9 (s), 159.1 (s), 158.5 (s), 154.8 (d), 131.2 (d), 131.2 (d), 124.1 (d), 123.1 (d), 116.0 (d), 116.0 (d), 106.0 (s), 99.9 (s), 94.5 (s). Lit. (Ganguly & Sarre, 1970) 270 $[M^+]$, 152, 118.

2,3-Dihydro-2-hydroxy-2,4-dimethyl-5-*trans*-propenylfuran-3-one (**5**): $[\alpha]^{20}_{D}$ = +1.75° (*c* = 0.29, EtOH); Lit. (Grove, 1971) $[\alpha]^{22}_{D}$ = ±2° (*c* = 0.5); UV (EtOH) λ_{max} (log ε) 310 (3.11), 285 sh (2.97), 227sh (3.47), 214 (3.64); Lit. (Grove, 1971) (ε) 312 (15000), 240 (9150), 215 (5850); IR (film) ν_{max} 3375, 2960, 2925, 1690, 1640, 1580 cm^{-1}; Lit. (Grove, 1971)ν_{max} 3340, 1680, 1640, 1575 cm^{-1}; ^{1}H NMR ppm 6.86 (dq, *J* = 15.6; 7 Hz, H-2'), 6.38 (dd, *J* = 15.6; 1.7Hz, H-1'), 4.01br (s, OH), 1.98 (dd, 7.0; 1.7Hz), 1.72 (3H, s), 1.55 (s); Lit. (Grove, 1971) ppm 6.85 (dq, *J* = 15.5; 6.5 Hz, H-2'), 6.3 (dd, *J* = 15; 1 Hz, H-1'), 4.85br (s, OH), 1.95 (dd, 6.5; 1 Hz), 1.68 (3H, s), 1.5 (3H, s). ^{13}C NMR ppm 202.5 (s), 176.9 (s), 139.7 (d), 118.9 (d), 106.9 (s), 101.9 (s), 22.2 (q), 19.0 (q), 5.4 (q). In the literature no ^{13}C NMR data were available. EIMS 168 $[M^+]$ (22), 156 (2), 125 (62), 97 (20), 83 (18), 79 (100).

4.4 New sesquiterpenoid secondary metabolites from the algicolous fungus *Drechslera dematioidea*

Abstract

From the inner tissue of the marine red alga *Liagora viscida* (Forskkal) C. Agardh the fungus *Drechslera dematioidea* was isolated. After mass cultivation the fungus was investigated for its secondary metabolite content and 10 new sesquiterpenoids (**6**-**8**, **10**-**15**, and **17**) were isolated. Compounds **13** and **15** exhibited antiplasmodial activity against *Plasmodium falciparum* strains K1 and NF54. The known compounds helminthosporol (**9**), *cis*-sativenediol (**16**), drechslerine E [(+)-secolongifolene diol] (**18**), isocochlioquinone A (**19**), isocochlioquinone C (**20**), and cochlioquinone B (**21**) were also isolated. All structures were elucidated using spectroscopic methods, mainly 1D and 2D NMR and MS.

Introduction

The genus *Drechslera* contains many terrestrial species that have been investigated for their natural products content, with ophiobolins being isolated from *D. oryzae*, a plant pathogen of rice (Sugawara *et al.*, 1988a), and from *D. maydis* and *D. sorghicola*, the causal agents of Southern corn leaf blight and leaf spot on Johnson grass (Sugawara *et al.*, 1987). Triticones A and B were obtained from the plant pathogenic fungus *D. tritici-repentis* (Sugawara *et al.*, 1988b), and eremophilane sesquiterpenes were isolated from the plant pathogen *D. gigantea* (Sugawara *et al.*, 1993), all indicating the genus *Drechslera* to have a very highly developed and diverse secondary metabolism. During our investigations related to the isolation, cultivation and screening of fungal strains associated with marine algae the phytopathogenic fungus *Drechslera dematioidea* was obtained from the marine red alga *Liagora viscida* (Forskkal) C. Agardh, collected from the Mediterranean Sea, Moraira, Spain. The antimicrobial activity and TLC information obtained from the EtOAc extract of the fungus as well as the fact that the secondary metabolite content of this fungus had never been investigated led us to the current investigation.

Materials and methods

Isolation and Taxonomy

Algal material was collected from the Mediterranean Sea, Moraira, Spain. After sterilization with 70 % EtOH for 40 seconds (optimal sterilization time for this alga) algal samples were rinsed with sterile sea water. Sterilized algae were then cut into small pieces and placed on agar plates containing isolation medium (15 g agar in 1000 mL sea water from the sample collecting site. After autoclaving 250 mg/mL of each the antibiotics benzylpenicillin and streptomycin sulphate were added by sterile filtration). Fungal colonies growing out of the algal tissue were transferred to medium for sporulation (1.0 g glucose, 0.1 g yeast extract, 0.5 g peptone from meat, enzymatic digest, 15 g agar, and 1000 mL sea water, pH 8) in order to enable isolates to be taxonomically identified.

Cultivation

The fungus was cultured at 20 °C for 30 days in 13.5 litres of solid medium containing 20 g/L biomalt extract, 6.8 g/L agar and demineralised water.

Biological activity, see chapter 3.5.

Extraction and Isolation

Prior to extraction with EtOAc (40 L) the solid medium and fungal mycelium were diluted with H_2O to enable them to be easily blended using the Ultra Turrax model T 25 at 8000 min^{-1}. The resultant EtOAc extract (10.0 g) was purified employing a combination of chromatographic techniques. First, it was passed over normal phase silica (vacuum liquid chromatography, VLC) using gradient elution from cyclohexane to EtOAc to MeOH to yield 15 fractions each of 250 mL.

VLC fractions 3 and 4 (2.4 g, eluted with cyclohexane:EtOAc 3:2), in which the antimicrobial and antialgal activity was concentrated, were combined and further subjected to normal phase VLC employing a gradient elution from petroleum ether to EtOAc to MeOH.

Fraction 4 (550 mg, eluted with petroleum ether:EtOAc 2:3) from this separation was purified by HPLC (LiChrocart 5 μm, 7 mm × 25 cm) using petroleum ether:$(CH_3)_2CO$ 82:18 as eluent and RP-18 HPLC (Eurospher RP-18 100, 5 μm, 8 mm × 25 cm) and MeOH:H_2O 76:24 as eluent yielding compounds **9**, **11**, **19**-**21**.

Fraction 3 [405 mg, eluted with petroleum ether:$(CH_3)_2CO$ 3:2] was further purified by HPLC (LiChrocart 5 μm, 7 mm × 25 cm) using petroleum ether:$(CH_3)_2CO$ 9:1 as eluent and RP-18 HPLC (Eurospher RP-18 100, 5 μm, 8 mm × 25 cm) using MeOH:H_2O 3:1 as eluent yielding compounds **6** and **18**.

VLC fractions 5 and 6 of the first separation which showed moderate antimicrobial activity, were combined (3.3 g, eluted with cyclohexane:EtOAc 15:85) and passed over normal phase silica (VLC) using a gradient elution from petroleum ether to EtOAc to MeOH.

Fraction 5 of this separation (1.2 g, eluted with petroleum ether:EtOAc 3:7) was purified by HPLC (LiChrospher Si 60 5 μm,7 mm × 25 cm) using petroleum ether:$(CH_3)_2CO$ 85:15 yielding compound **15**.

VLC fractions 7, 8 and 9 were combined on the basis of their similar TLC information (2.9 g, eluted with EtOAc:MeOH 4:1) and further investigated due to their interesting ^1H NMR data. They were subjected to further normal phase VLC using a gradient elution from petroleum ether to EtOAc to MeOH. The third fraction of this separation (350 mg, eluted with 100 % EtOAc) was further separatedusing normal phase HPLC (LiChrospher Si 60 5 μm, 7 mm × 25 cm) using petroleum ether:$(CH_3)_2CO$ 74:26 as eluent. Resultant fractions were further purified by RP-18 HPLC (Eurospher RP-18 100 5 μm, 8 mm × 25 cm) using MeOH:H_2O 7:3 or 65:35 as eluent to yield compounds **7**, **8**, **10**, **12**-**14**, **16**, and **17**.

Results and Discussion

The fungus *Drechslera dematioidea* was cultivated on a solid biomalt medium. Successive fractionation of the EtOAc extract by vacuum-liquid chromatography (VLC) and normal- and reversed-(RP-18) phase HPLC yielded sixteen compounds **6**-**21**, thirteen of which (**6**-**18**) are sesquiterpenoids, with the remaining three (**19**-**21**)

being of mixed biosynthetic origin containing a p-benzoquinone ring linked to a sesquiterpene moiety and to a C_7 side-chain.

Drechslerine A (**6**) has the molecular formula $C_{15}H_{24}O_3$ as deduced by FABMS, GC-MS analysis of a TMS derivative and accurate mass measurement. From its ^{13}C NMR spectroscopic data it was evident that one of the four elements of unsaturation, indicated by the molecular formula of **6**, could be attributed to an *exo*-methylene group [δ 105.5 (C-12, t) 156.9 (C-2, s)] as the only multiple bond within the molecule; drechslerine A is thus tricyclic. The ^1H and ^{13}C NMR spectra showed the presence of two further methylene groups [δ 20.7 (C-5, t), 39.1 (C-4, t)], six methine groups [δ 46.3 (C-7, d), 48.8 (C-6, d), 56.6 (C-13, d), 59.6 (C-1, d)], two of them attached to oxygen [δ 76.0 (C-15, d); 78.6 (C-14, d)], three methyl groups [δ 1.04 (H_3-8, s), 1.33 (H_3-10, s), 1.27 (H_3-11, s)], all adjacent to quaternary carbons [δ 41.5 (C-3, s), 74.0 (C-9, s)], one of which is bonded to oxygen. These data also showed that all but three of the hydrogens were bonded directly to carbons indicating the remaining three to be present as part of hydroxyl functions. After assignment of all protons to their directly bonded carbons *via* a one-bond ^1H-^{13}C shift correlated 2D NMR measurement (HMQC), it was possible to deduce from the ^1H-^1H COSY spectrum of **6** that H_2-4 couples with H_2-5, H_2-5 with H-6, H-6 also with H-7, H-7 also with H-13 which also couples with H-14, H-14 further couples with H-1, which also couples with H-15, which in turn couples with H-7. These couplings indicated C-C bonds to occur between C-4 and C-5, C-5 and C-6, C-6 and C-7, C-7 and C-13, C-13 and C-14, C-14 and C-1, C-1 and C-15, and C-15 and C-7, and completed the first two rings within the molecule. Diagnostic long-range ^1H-^{13}C HMBC correlations observed from the resonance of H_3-8 to those of C-2, C-3, C-4 and C-13 showed C-8 to bond to the quaternary carbon C-3 which further bonded to C-2, C-4 and C-13. The long-range correlations observed between the resonances for H_2-12 and that of C-1, indicated C-1 and C-2 to bond directly, and enabled the third ring within **6** to be completed. Further long-range correlations, this time observed between the resonances for H_3-10 and H_3-11 and those of C-6 and C-9 showed the two methyl groups to reside on the quaternary carbon C-9, which is further bonded to C-6. Remaining to be incorporated into the planar structure were the hydroxyl functions. On the basis of the ^{13}C NMR chemical shifts of C-9 (δ 74.0, s), C-14 (δ 78.6, d) and

C-15 (δ 76.0, d) it was evident that the hydroxyl functions must be located at C-9, C-14 and C-15. As one of the two protons located at C-5 (H-5ax) had three large coupling constants (> 10 Hz), and one small one (< 5 Hz) it had to have an axial orientation. This deduction also meant that H-6 had to be axial and on the opposite side of the molecule to H-5α, and thus gave the six-membered ring a chair conformation. As H-6 has only one coupling constant indicative of an axial-axial interaction, with H-5, H-7 was given an equatorial orientation, and in so doing placed the C-7→ C-15→ C-1→ C-2→ C-3 part of **6** on the under face of the molecule. This deduction also meant the methyl group at C-3 had to be equatorial and H-13 had to be axial. The relative configuration at C-15 was deduced to be as shown in **6** based on $J_{\text{H-7, H-15}} < 2$ Hz, which meant these two protons must be at almost 90° to one another. The deduced relative stereochemistry of **6** and the shown configuration at C-14 was further supported by NOE difference measurements. Thus, irradiation at the resonance frequency of H-1 caused enhancement of the resonance of H-14, one part of H_2-12 and H-15. Irradiation at the resonance frequency of H-7 gave enhancement for the resonance of H-13 and H-6. Irradiation at the resonance frequency of H_3-10 and H_3-11 caused enhancement of the resonance for H-15. Finally, irradiation at the resonance frequency of H_3-8 gave enhancement of one of the resonances associated with H_2-12, one of the H_2-4 resonances, and the resonances associated with H-13 and H-14. Thus, the relative stereochemistry for drechslerine A (**6**) is best described as 1 R^*, 3 S^*, 6 R^*, 7 S^*, 13 R^*,14 R^*, 15 S^*.

Drechslerine A seems to be identical in structure to the triol reported by Dorn and Arigoni (1975a). The total lack of any spectroscopic data in the work by these authors, however, makes any reasonable comparison between the two structures impossible, the two compounds also have significantly different optical rotations (-2.0° for **6** as compared to +11° for the compound reported by Dorn and Arigoni).

Table 13. ^{13}C NMR data for compounds **6**-**18**; compounds **6**, **7**, **9**, **12**, **15**-**18** were measured in CD_3OD, and compounds **8**, **10**, **11**, **13, 14** in $CDCl_3$.

position	**6**[b]	**7**[a]	**8**[b]	**9**[b]	**10**[b]	**11**[b]	**12**[b]	**13**[b]	**14**[a]	**15**[b]	**16**[a]	**17**[c]	**18**[a]
1	59.6 d[d]	124.2 d	134.9 s	137.3 s	139.1 s	n.o.[e]	38.2 d	36.8 d	51.9 d	56.6 d	55.8 d	52.9 d	128.3 d
2	156.9 s	147.1 s	180.9 s	165.9 s	170.7 s	55.0 d	50.5 d	52.3 d	51.6 d	89.0 s	159.3 s	68.4 s	147.9 s
3	41.5 s	47.7 s	47.8 s	50.8 s	52.2 s	41.9 s	45.1 s	44.2 s	42.2 s	49.4 s	44.0 s	39.9 s	51.4 s
4	39.1 t	35.8 t	35.6 t	34.2 t	34.9 t	36.2 t	41.3 t	41.7 t	37.7 t	30.5 t	41.2 t	37.3 t	45.7 t
5	20.7 t	26.4 t	27.0 t	25.2 t	21.8 t	26.0 t	21.1 t	21.5 t	26.0 t	24.1 t	26.8 t	26.0 t	22.1 t
6	48.8 d	45.4 d	45.3 d	44.9 d	49.8 d	50.2 d	49.8 d	50.9 d	47.0 d	44.9 d	43.7 d	43.4 d	42.7 t
7	46.3 d	43.9 d	41.3 d	41.3 d	40.7 d	51.4 d	52.5 d	50.5 d	41.1 d	39.5 d	43.0 d	42.5 d	36.7 s
8	28.8 q	19.0 q	17.8 q	18.4 q	18.6 q	22.0 q	19.8 q	20.2 q	18.8 q	20.9 q	21.1 q	19.6 q	21.8 q
9	74.0 s	33.8 d	33.7 d	31.8 d	72.9 s	30.0 d	72.6 s	73.2 s	32.2 d	30.6 d	34.3 d	34.5 d	58.9 d
10	28.9 q	21.6 q	21.8 q	21.7 q	28.8 q	21.5 q	29.3 q	28.9 q	21.4 q	20.0 q	21.5 q	21.5 q	32.5 q
11	27.0 q	21.3 q	21.0 q	20.7 q	28.3 q	20.4 q	28.6 q	28.8 q	21.2 q	18.1 q	21.3 q	21.3 q	27.9 q
12	105.5 t	59.9 t	69.3 t	10.6 q	11.0 q	62.1 t	69.5 t	64.9 t	71.6 t	61.6 t	103.3 t	51.1 t	59.7 t
13	56.6 d	63.9 d	68.1 d	61.3 d	64.5 d	50.7 d	152.6 s	157.8 s	67.8 s	52.5 d	59.7 d	59.6 d	47.0 d
14	78.6 d	62.7 t	61.9 t	62.5 t	61.8 t	6.3 q	105.8 t	100.9 t	51.0 t	69.0 t	76.0 d	69.9 d	35.2 t
15	76.0 d	-	173.8 s	188.2 d	192.5 d	-	171.6 s	103.8 d	174.5 s	203.3 d	70.0 d	73.2 d	62.1 t
16	-	-	-	-	-	-	-	54.7 q	-	-	-	-	-

All assignments are based on extensive 1D and 2D NMR measurements (COSY, HMQC, HMBC).
[A] Spectra were recorded at 75.5 MHz.
[b] Spectra were recorded at 100 MHz.
[c] Spectra were recorded at 150 MHz.
[d] Implied multiplicity by DEPT (C = s, CH = d, CH_2 = t, CH_3 = q).
[e] n.o. = not observed.

Table 14. ^{1}H NMR data for compounds **6**-**18**; compounds **6**-**8**, **10**, **14**, **16**-**18** were measured in CD_3OD, compounds **9**, **11**, **12**, **13**, **15** in $CDCl_3$.

pos.	**6**[b]	**7**[a]	**8**[c]	**9**[c]	**10**[a]	**11**[b]
1	2.68 (brs)	5.59 (brd, 1.5)	-	-	-	-
2	-	-	-	-	-	1.69 (dd, 5.1, 8.1)[d]
4	1.38 (ddd, 1.5, 13.0, 13.2)	1.43 (m)	1.60 (m)	1.41 (m)	1.55 (m)	1.41 (ddd, 5.6, 5.9, 13.7)[d]
	1.75 (ddd, 3.1, 3.1, 13.2)	1.29 (m)	1.63 (m)			1.65 (ddd, 1.5, 7.6, 13.7)[d]
5	1.19 (dddd, 1.5, 3.1, 3.1, 13.0)	1.68 (m)	1.94 (dddd, 3.5, 3.5, 3.6, 13.3)	1.75 (m)	1.76 (m)	1.79 (dddd, 1.5, 5.6, 5.6, 14.2)[d]
	1.64 (dddd, 3.1, 12.8, 13.0, 13.0)	1.24 (m)	0.92 (m)	0.88 (m)	0.97 (m)	0.86 (dddd, 5.9, 7.6, 13.6, 14.2)[d]
6	1.51 (ddd, 3.1, 3.1, 12.8)	1.09 (m)	1.20 (m)	0.99 (m)	1.54 (m)	1.31 (dddd, 2.5, 5.6, 10.7, 13.6)
7	2.62 (brm)	2.80 (brs)	3.20 (brs)	3.19 (brs)	3.27 (brs)	2.68 (s)
8	1.04 (s)	1.00 (s)	1.20 (s)	1.04 (s)	1.09 (s)	1.08 (s)
9	-	1.28 (m)	1.20 (m)	0.98 (brs)	-	1.49 (m)[d]
10	1.33 (s)	0.99 (d, 6.8)	1.10 (d, 6.1)	1.08 (d, 5.4)	1.34 (s)	1.01 (d, 6.6)
11	1.27 (s)	0.89 (d, 6.8)	0.90 (d, 6.1)	0.77 (d, 5.4)	1.16 (s)	0.81 (d, 6.6)
12	5.03 (s)	4.04 (dd, 1.5, 14.3)	4.97 (dd, 1.3, 18.3)	2.02 (s)	2.12 (s)	3.84 (dd, 5.1, 10.7)
	4.77 (s)	4.12 (ddd, 1.0, 1.1, 14.3)	4.90 (d, 18.3)			3.49 (dd, 8.1, 10.7)
13	1.78 (m)[d]	1.58 (dd, 5.3, 9.4)	2.05 (dd, 5.1, 9.7)	1.68 (dd, 5.4, 8.7)	1.77 (m)	2.09 (brq, 7.1)
14	4.31 (m)	3.68 (dd, 5.3, 10.6)	3.76 (dd, 5.1, 11.2)	3.65 (dd, 5.4, 10.8)	3.64 (dd, 4.6, 10.7)	0.95 (d, 7.1)
		3.41 (dd, 9.4, 10.6)	3.39 (dd, 9.7, 11.2)	3.33 (dd, 8.7, 10.8)	3.23 (dd, 9.7, 10.7)	
15	3.75(brs)	-	-	10.0 (s)	9.98 (s)	-

position	**12**[b]	**13**[c]	**14**[c]	**15**[b]	**16**[a]	**17**[c]	**18**[a]
1	2.61 (brs)	2.73 (brs)	2.49 (d, 2.5)	2.84 (d, 1.5)	2.62 (brs)	1.69 (brs)	5.75 (brs)
2	1.77 (brdd, 1.7, 4.5)	1.31 (d, 1.5)	2.08 (dd, 2.5, 4.5)	-	-	-	-
4	1.58 (m)	1.47 (m)	1.67 (m)	1.20 (m)[d]	1.55 (m)	1.49 (m)	1.71 (m)
	1.53 (m)		1.49 (m)		1.38 (m)	1.40 (m)	1.42 (m)
5	1.69 (m)	1.59 (m)	1.89 (m)	1.15 (m)[d]	1.64 (m)	1.70 (m)	1.65 (m)
	1.52 (m)	1.47 (m)	1.52 (m)	1.67 (m)[d]		1.65 (m)	1.39 (m)
6	1.66 (m)	1.45 (m)	1.51 (m)	1.23 (m)[d]	1.36 (m)	1.50 (m)	1.42 (m)
7	3.80 (brs)	2.95 (brs)	2.56 (brs)	2.66 (brs)	2.54 (brs)	2.58 (brs)	-
8	1.23 (s)	1.21 (s)	0.98 (s)	0.97 (s)	1.09 (s)	0.85 (s)	1.06 (s)
9	-	-	1.46 (m)	1.63 (m)[d]	1.39 (m)	1.45 (m)	1.99 (brs)
10	1.27 (s)	1.25 (s)	1.01 (d, 2.3)	0.81 (d, 6.1)	1.00 (d, 6.2)	1.01 (d, 6.5)	0.97 (s)
11	1.24 (s)	1.21 (s)	0.99 (d, 2.3)	0.89 (d, 6.1)	0.93 (d, 6.2)	0.97 (d, 6.5)	0.98 (s)
12	4.42 (dd, 0.5, 11.7)	3.77 (dd, 1.5, 11.1)	4.59 (d, 11.9)	3.88 (m)[d]	4.95 (s)	2.79 (d, 4.6)	4.00 (ddd, 1.5, 1.7, 14.9)
	4.25 (dd, 4.7, 11.7)	3.74 (dd, 2.0, 11.1)	4.39 (dd, 4.5, 11.9)		4.65 (s)	2.74 (d, 4.6)	4.07 (ddd, 1.7, 1.9, 14.9)
13	-	-	-	1.63 (m)[d]	1.56 (brs)	1.62 (brs)	2.02 (m)
14	5.20 (dd, 0.8, 1.0)	4.83 (brs)	2.92 (d, 4.5)	3.87 (d, 7.6)	3.57 (dd, 0.9, 6.2)	4.15 (d, 6.5)	1.76 (m)
	4.86 (dd, 1.0, 1.0)	4.73 (brs)	2.74 (d, 4.5)	3.46 (d, 7.6)			1.32 (m)
15	-	4.36 (d, 3.6)	-	9.95 (d, 1.5)	3.99 (dd, 0.9, 6.2)	3.94 (d, 6.5)	3.65 (m)
							3.56 (m)
Other	-	3.36 (s, OCH_3-16)	-	2.24 (br, 12-OH)	-	-	-

[a] Spectra were recorded at 300 MHz.
[b] Spectra were recorded at 400 MHz.
[c] Spectra were recorded at 600 MHz.
[d] Chemical shifts were obtained from HMQC spectrum.

From accurate mass measurement drechslerine B (**7**) was found to have the molecular formula $C_{14}H_{24}O_2$. Its ^{13}C NMR spectrum contained 14 signals (see Table 13). This data also indicated the molecule to have only one multiple bond, a carbon-carbon double bond [δ 124.2 (C-1, d), 147.1 (C-2, s)], and showed the two remaining elements of unsaturation in the molecule to be in the form of rings. As all, except two protons, could be associated with directly bonded carbon atoms *via* a HMQC measurement it was evident that the oxygen atoms within **7** must be present in the form of two hydroxyl functions. The ^1H and ^{13}C NMR spectra further revealed the presence of four methylene groups, two of them attached to the hydroxyl groups [δ 59.9 (C-12, t), 62.7 (C-14, t), (ν 3300 cm^{-1})], four methine groups [δ 33.8 (C-9, d), 43.9 (C-7, d), 45.4 (C-6, d), 63.9 (C-13, d)], three methyl groups, [δ 0.89 (H_3-11, d), 1.00 (H_3-8, s), 0.99 (H_3-10, d)], and a further quaternary carbon [δ 47.7 (C-3, s)]. From the ^1H-^1H COSY it was possible to deduce the majority of the planar structure of compound **7**. Thus, ^1H-^1H COSY cross-peaks between the resonances for H_3-10 and H_3-11 and the resonances for H-9, between the resonance for H-9 and that for H-6, between the resonance for H-6 and those for H_2-5, and between the resonances for H_2-5 and those of H_2-4, were observed. H-6 also demonstrated coupling to H-7, which further coupled with H-1 and H-13, H-13 in turn coupled with H_2-14. These ^1H-^1H COSY couplings revealed C-1 to bond with C-7 which bonds with C-13 and C-6, C-13 to bond with C-14, C-6 to further bond with C-9 and C-5, C-9 to bond with C-10 and C-11, and C-5 to also bond with C-4. Diagnostic long-range ^1H-^{13}C 2D NMR correlations between the resonances of H_2-12 and those of C-1 and C-2 showed C-12 to bond to C-2. Further long-range correlations observed between the resonance of CH_3-8 and those of C-2, C-3, C-4 and C-13 clearly positioned H_3-8 at C-3, and showed C-3 to also bond with C-2, C-4 and C-13 and in so doing enabled the two rings within **7** to be completed. The two secondary hydroxyl functions were thus CH_2OH-12 and CH_2OH-14, a deduction supported also by their ^1H and ^{13}C NMR chemical shifts. With the planar structure of **7** established the relative configuration at the four chiral centres within the molecule remained to be determined primarily from the results of NOE difference measurements. Thus, irradiation at the resonance frequency of H_3-11 caused enhancement of the resonances for H-1 and H-7. NOEs were also detected between H-7 and H-1, and H-7 and H_2-14. These NOE data, and the facts that the coupling constant between H-7 and H-13 is less than 3 Hz, and that

H-1 and H-13 “W“ couple, are consistent with the relative configuration of **7** being described as 3 R^*, 6 R^*, 7 R^*, 13 S^*.

Drechslerine C (**8**) was analysed for $C_{15}H_{22}O_3$ by accurate mass measurement. The ^{13}C NMR spectral data of **8** were very similar (see Table 13) to those found for compound **7**, it was thus concluded that the two compounds were closely related. Careful examination of all of the spectroscopic data of **7** and **8** indicated the differences between the two data sets to arise from the presence of an extra carbon (C-15) in **8** which was assigned to an α,β unsaturated lactone function (δ 173.8, s), a deduction which was also supported by both the IR (1775 cm^{-1}) and UV (λ_{max} 235 nm) data of **8**. The electron withdrawing effects of the lactone function significantly influences the chemical shifts of C-1 (δ 134.9, s), C-2 (δ 180.9, s) and C-12 (δ 69.3, t) relative to the equivalent centres in **7** (δ 124.2, d; 147.1, s; 59.9, t), respectively. These data also showed the ester function to be between C-1 and C-12. NOE difference measurements similar to those made for compound **7**, as well as coupling constant analyses, revealed **7** and **8** to have identical relative stereochemistries.

Accurate mass measurement showed **9** to have the molecular formula $C_{15}H_{24}O_2$. Comparison of its ^1H NMR (Pena-Rodriguez *et al.*, 1988), UV, and IR data and optical rotation (Tamura *et al.*, 1963), with published values enabled **9** to be identified as the known compound helminthosporol or a stereoisomer thereof. As helminthosporol was only partially characterized in the literature 1D and 2D NMR experiments together with NOE difference measurements allowed the complete structure to be deduced as shown in **9**. Since the optical rotation of our metabolite and the one reported for helminthosporol (Tamura *et al.*, 1963) are essentially identical it was concluded that **9** is indeed helminthosporol. The first reported isolation of helminthosporol was from the culture broth of *Helminthosporium sativum* (Tamura *et al.*, 1963). This compound is probably most noted for its ability to inhibit Acyl-CoA cholesterol acyltransferase (ACAT) (Park *et al.*, 1993).

A compound similar in structure to **9** was also obtained from the EtOAc extract of *D. dematioidea*. This compound, 9-hydroxyhelminthosporol (**10**), differs from **9** by the

presence of a hydroxyl group at C-9 as evidenced by the ^{13}C chemical shift and multiplicity of C-9 (δ 72.9, s) when compared to corresponding carbon in **9** (δ 31.8). All other NMR data for the two compounds were in good agreement with each other even though they were obtained in different solvents. In a relative sense, the two molecules were deduced to have the same stereochemistry on the basis of the similarities between the two NMR data sets, in particular the fact that all of the couplings associated with H-7 are between 0 and 2 Hz.

Drechslerone A (**11**) was found to have the molecular formula $C_{14}H_{24}O_2$ by accurate mass measurement. Its ^{13}C NMR spectrum contained only 13 resonances (see Table 13), the resonance for the carbonyl group, C-1, not being observed, but this functionality was clearly present as evidenced by the strong 1730 cm^{-1} absorption in the IR spectrum of **11**. From all the spectroscopic data of **11** only one multiple bond, the C=O group, was evident, indicating the molecule to be bicyclic, and the remaining oxygen within the molecule to be present in the form of an OH group. The ^{1}H and ^{13}C NMR spectra further revealed the presence of three methylene groups [δ 26.0 (C-5, t), 36.2 (C-4, t)], one of them attached to the hydroxyl group [δ 62.1 (C-12, t)], five methine groups [δ 30.0 (C-9, d), 50.2 (C-6, d), 50.7 (C-13, d), 51.4 (C-7, d), 55.0 (C-2, d)], and four methyl groups [δ 0.81 (H_3-11, d), 0.95 (H_3-14, d), 1.01 (H_3-10, d)], one of them [δ 1.08 (H_3-8, s)] attached to a quaternary carbon [δ 41.9 (C-3, s)]. From the ^{1}H-^{1}H COSY spectrum of **11** coupling between H_3-10, H_3-11 and H-9, H-9 and H-6, H-6 and H-7, H-7 and H-13, H-13 and H_3-14 were observed, and showed C-10 and C-11 to be bonded to C-9, C-9 to C-6, C-6 to C-7, C-7 to C-13 and C-13 to C-14. In the ^{1}H-^{1}H COSY spectrum coupling was also seen between H-6 and H_2-5, H_2-5 and H_2-4, and revealed C-6 to bond to C-5, and C-5 to also bond to C-4. A second fragment of **11** was deduced from the ^{1}H-^{1}H coupling of H-2 to H_2-12 which revealed C-2 to be bonded to C-12. From diagnostic ^{1}H-^{13}C long-range correlations (HMBCs) between the resonance for H_3-8 and those of C-2, C-3, C-4, and C-13 it was evident that the methyl group CH_3-8 bonded to C-3 which further bonded to C-2, C-4 and C-13, thus completing one of the two rings within **11**. This only left the carbonyl group to be attached to C-2 and C-7 which completed the second ring within the molecule and also its planar structure, and left the relative configuration at C-2, C-6, C-7 and C-13 to be established. As all of the coupling constants associated

with H-7 were less than 2 Hz it was evident that it must be almost at 90° to both H-6 and H-13 giving the molecule the same relative configurations at C-3, C-6 and C-7 to those shown for compounds **7**-**10**. An NOE interaction between H-13 and one of the protons at C-12 enabled the relative stereochemistry at C-2 and C-13 to be resolved as shown in **11**.

Mass spectral analysis of **12**, drechslerone B, indicated it to have the molecular formula $C_{15}H_{22}O_3$. Its ^{13}C NMR spectrum contained 15 resonances (see Table 13) of which three were associated with multiple bonds; an ester carbonyl function [δ 171.6 (C-15, s)] which was also seen in the IR spectrum (1730 cm^{-1}), and an *exo*-methylene group [δ 152.6 (C-13, s), 105.8 (C-14, t)]; the molecule is thus tricyclic. The ^{1}H and ^{13}C NMR spectra together revealed the presence of three further methylene groups [δ 21.1 (C-5, t), 41.3 (C-4, t)], one of them attached to oxygen [δ 69.5 (C-12, t)], four methine groups [δ 38.2 (C-1, d), 49.8 (C-6, d), 50.5 (C-2, d), 52.5 (C-7, d)], three methyl groups [δ 1.23 (H_3-8, s), 1.24 (H_3-11, s), 1.27 (H_3-10, s)], and two further quaternary carbons [δ 45.1 (C-3, s)], one of them attached to oxygen [δ 72.6 (C-9, s)]. The remaining oxygen in the molecule is in the form of a hydroxyl function. In the ^{1}H-^{1}H COSY spectrum cross-peaks between the resonances for H_2-12 and that of H-2, and between those of H-2 and H-1, H-1 and H-7, H-7 and H-6, H-6 and H_2-5, and H_2-5 and H_2-4 were observed, and enabled the C-1 to C-12, *via* C-7, part of the molecule to be deduced. Long-range ^{1}H-^{13}C correlations from the resonances for H_2-12 to that for C-15 enable the lactone to be positioned between C-12 and C-15. Further long-range ^{1}H-^{13}C correlations from the resonance for H-7 to those of C-13 and C-15, and from the resonances for H_2-14 to those of C-3 and C-7 showed C-13 to bond to C-3 and C-7, and C-1 to bond to C-15, and so completed the two remaining rings within the molecule. Further, the isopropyl group was identified and positioned; the resonances for the methyl groups H_3-10 and H_3-11 showed HMBC correlations to the resonances for C-9 and C-6. As both methyl groups are tertiary they must bond to C-9. The chemical shift of C-9 (δ 72.6) which bonds to C-6 showed the hydroxyl group also to reside at C-9. Further long-range correlations from the resonance for H_3-8 to those for C-2, C-3, C-4 and C-13 enabled the connection from C-8 to C-3, which is further bonded to C-2, C-4 and C-13, to be established, and hence the basic structure of the molecule to be completed. The

results of NOE difference measurements and coupling pattern analyses then allowed the relative configuration of the molecule to be deduced as shown in **12**. Thus, irradiation at the resonance frequency of H-1 caused enhancement of the resonance for H-2, and *vice versa*, and showed both protons to be positioned on the same side of the molecule. As irradiation at the resonance frequency of H-1 also caused enhancement of the resonances for H-5, H-7, H_3-10 and H_3-11 it was evident that H-1, H-2 and the isopropanoyl group had to be α-oriented, and that the C-7, C-13, C-3 bridge was β-oriented.

The MS, and ^{1}H and ^{13}C NMR chemical shift data of drechslerine D (**13**) revealed it to be the 15-methoxyl derivative of **12**. The orientation of the methoxyl group was deduced as being α on the basis of the NOE interaction observed between H-7 and H-15.

The spectroscopic data of **14** (drechslepoxide A) indicated it to have a structure very similar to those of **12** and **13**. The major differences between the three data sets being attributable to an *exo*-epoxy group in **14** [δ 67.8 (C-13, s), 51.0 (C-14, t)] instead of the *exo*-methylene group present in **12** and **13**, and the absence, in **14**, of a hydroxyl group at C-9. Low power irradiation at the resonance frequencies of H_3-10 and H_3-11 caused enhancement of the resonances of H-1, H_2-5 and H-7 and showed the isopropyl group, H-1 and H-7 to have the same relative orientation as in **12** and **13**. Low power irradiation at the resonance frequency for H_3-8 caused enhancement of the resonances of H-2, H_2-4, H-12 (δ 4.59) and H-14 (δ 2.92), and showed the *exo*-epoxy group to have the orientation as shown in **14**. The ^{1}H-^{1}H coupling information also supported these deductions.

Drechsleral (**15**) was analysed for $C_{15}H_{24}O_3$ by accurate mass measurement. Of the four elements of unsaturation indicated by the molecular formula of **15** one was attributed to an aldehyde group [δ 203.3 (d, C-15)] which also accounted for all of the multiple bonds within the molecule; **15** is thus tricyclic. From its IR spectrum the presence of the aldehyde group (1715 cm^{-1}) was supported and a hydroxyl functionality (3440 cm^{-1}) was evident. The remaining oxygen in the molecule had, thus, to be present as an ether function. In the ^{1}H-^{1}H COSY spectrum cross-peaks

observed between the resonances for H_3-10 and H_3-11 and that for H-9, between that for H-9 and that for H-6, between that for H-6 and those for H_2-5, between those for H_2-5 and those for H_2-4, showed C-10 and C-11 to be bonded to C-9 which is further bonded to C-6, C-6 to C-5, and C-5 to C-4. Further ^{1}H-^{1}H COSY cross-peaks between the resonance for H-6 to that for H-7, between that for H-7 to that for H-1, and between that for H-1 to that for H-15, revealed C-6 to bond also to C-7, C-7 to C-1, and C-1 to the aldehyde carbon, C-15. Additionally, ^{1}H-^{1}H COSY cross-peaks were present which showed H-7 to couple with H-13, and H-13 with H_2-14 and revealed C-7 to bond also to C-13, and C-13 to further bond with C-14. The hydroxyl group proton (δ 2.24 brm) couples with H_2-12 (δ 3.88), and clearly showed this function to reside at C-12. Consequently, C-2 (δ 89.0, s) and C-14 (δ 69.0, t), which are both attached to oxygen as evidenced by their chemical ^{13}C NMR shifts, are both bonded to oxygen of the ether moiety. Diagnostic long-range ^{1}H-^{13}C 2D NMR correlations observed between the resonances of H_3-8 and those for C-2, C-3, C-4 and C-13 showed C-8 to be bonded to C-3 which is further bonded to C-2, C-4 and C-13, and in so doing completed two of the three rings within the molecule. Further long-range correlations, this time seen between the resonances for H_2-12 and those for C-1 and C-2, showed C-12 to bond to C-2 which further bonded to C-1, and thus completed the third and final ring and the planar structure of **15**. On the basis of the magnitude of ^{1}H-^{1}H coupling constants, particulary those between H-7 and H-1, H-7 and H-6, and H-7 and H-13 (< 2 Hz), and also the rigidity of the molecule, the relative configuration for **15** is proposed as that shown.

Comparison of the ^{1}H NMR data and optical rotation for **16** (see Table 14) with those published for *cis*-sativenediol, a plant growth promoter (Nukina *et al.*, 1975), showed the two compounds to be identical. This deduction was also supported by the ^{13}C NMR (see Table 13) and MS data of **16**.

MS analysis of drechslepoxide B (**17**) showed it to have the molecular formula $C_{15}H_{24}O_3$. Comparison of its ^{1}H and ^{13}C NMR spectral data with that of **16** revealed the two compounds to be structurally very similar. Evident from the ^{1}H and ^{13}C NMR data of **17** was the absence of any resonances associated with an *exo*-methylene group and the presence of resonances attributable to an *exo*-epoxy function in its

place. All of the remaining physical and spectroscopic data showed the remaining structural features of the two molecules to be identical, in a relative sense. The relative configuration at C-2 was deduced as being as shown in **17** on the basis of the NOEs observed between H_3-8 and the epoxy-proton (δ 2.79) resonance, and between the other epoxy-proton (δ 2.74) and H-15. These NOE interactions also further supported the overall relative configurations shown for **16** and **17**.

Mass spectral analysis of drechslerine E (**18**) showed it to have the molecular formula $C_{15}H_{26}O_2$. All of the remaining data for this compound (^{1}H and ^{13}C NMR both 1D and 2D) clearly showed **18** to be a compound identical to the one reported by Dorn and Arigoni (1974), and the optical antipode of the one synthesized by Yadav *et al.* (1983).

Together with compounds **6**-**18**, isocochlioquinones A and C (**19** and **20**), and cochlioquinone B (**21**) were also isolated from the EtOAc extract of *D. dematioidea*. They were identified by comparison of their ^{1}H and ^{13}C NMR data with published values (Miyagawa *et al.*, 1994, Lim *et al.*, 1998). Isocochlioquinone A was first isolated from a culture of *Bipolaris bicolor* EI-1, a fungal pathogen of gramineous plants. Isocochlioquinone C, also a plant fungal pathogen, was also first isolated from a culture of *Bipolaris*, this time *B. cynodontis* cynA. Culture filtrates of the perfect stage of *B. oryzae*, *Cochliobolus miyabeanus*, were the original source of cochlioquinone B (Carruthers *et al.*, 1971; Canonica *et al.*, 1976), which has also been isolated from *B. bicolor* (Miyagawa *et al.*, 1994).

Studies into the biosynthetic origin of the carbon skeleton of the cochlioquinones showed their mixed biosynthesis occurred by the introduction of a farnesyl unit onto an aromatic precursor whose secondary methyl groups derived from methionine (Canonica *et al.*, 1980). All three cochlioquinone derivatives inhibit the root growth of gramineous plants and electron transfer in the mitochondrial respiratory system (Lim *et al.*, 1998).

From a marine-derived strain of the fungus *D. dematioidea* ten new sesquiterpenes (**6**-**8**, **10**-**15**, **17**) were obtained. Drechslerine A (**6**) is biosynthetically most likely to be

a sativan derivative, with **7**-**10** being likely copacamphan derivatives. The secosativan-skeleton is represented by **11**, and the sativan-skeleton by **16** and **17**. Drechslerine E [(+)-secolongifolene diol] (**18**) is the sole representative of a secolongifolan carbon-skeleton based compound. Clearly, all of these isolates are congeneric products in that they have a similar biosynthetic origin. The fungi *Helminthosporium victoriae* and *H. sativum* (Dorn *et al.*, 1975b) contain natural products similar to compounds **1**-**13**.

All compounds were tested for their antimicrobial and antialgal activities in agar diffusion assays, in ELISA based assays in order to evaluate their HIV-1 reverse transcriptase and tyrosine kinase ($p56^{lck}$) inhibitory activity, in assays to investigate their activities against brine shrimps, nematodes, and *Mycobacterium tuberculosis* as well as for their antiplasmodial activity. The results of these investigations are presented in Table 15. Notable activities were observed for **13** and **15** which both exhibited antiplasmodial activity against two different strains of *Plasmodium falciparum*. Drechslerine D (**13**) showed activities against *P. falciparum* K1 (IC_{50} = 5.1 μg/mL) and NF 54 (IC_{50} = 3.7 μg/mL), while drechsleral (**15**) exhibited antiplasmodial activity against *P. falciparum* K1 (IC_{50} = 2.9 μg/mL) and NF 54 (IC_{50} = 4.2 μg/mL), respectively. Compound **15** also reduced the activity of the enzyme tyrosine kinase $p56^{lck}$ to 72 % at a concentration of 200 μg/mL. A significant inhibition of this enzyme was obtained with cochlioquinone B (**21**); 48 % residual enzyme activity at a compound concentration of 40 μg/mL. Additionally, **21** was antiplasmodial against *P. falciparum* K1 (IC_{50} = 2.6 μg/mL) and NF 54 (IC_{50} = 3.4 μg/mL). The known compound helminthosporol (**9**), reduced the activity of the enzyme tyrosine kinase $p56^{lck}$ to 58 % at a concentration of 200 μg/mL. The same enzyme was also inhibited by the known compounds *cis*-sativenediol (**16**) which reduced it to 58 %, and isocochlioquinone C (**20**) which reduced it to 44 %, both at a concentration of 200 μg/mL. In the assays which investigated activity against brine shrimps, nematodes, and *M. tuberculosis* all of the tested compounds appeared to be inactive.

Table 15. Antibacterial, antifungal, enzyme inhibitory (reverse transcriptase, tyrosine kinase) and antiplasmodial activities of compounds **6**-**21**.

	Antimicrobial activity		Enzyme inhibitory activity			Antiplasmodial activity (IC_{50})	
Compound	Bacteria[a] [50 µg/disc]	Fungi[b] [50 µg/disc]	TK^{lck} activity[c] [200 µg/mL]	TK^{lck} activity[c] [40 µg/mL]	RT activity[d] [66 µg/mL]	K1 [e] [ng/mL]	NF54 [e] [ng/mL]
6	n.a.	n.a.	79.9	-	80.9	> 10000	> 10000
7	Ec 1mm	Mv 3mm; Er 2 mm	> 100	-	93.9	> 10000	> 10000
8	n.a.	Mv 2 mm	> 100	-	> 100	> 10000	> 10000
9	Bm 1mm	Mv 2 mm	58.2	-	> 100	4711	6705
10	n.a.	Mv 1 mm	> 100	-	80.9	> 10000	> 10000
11	n.a.	Mv 1 mm	-	-	90.2	-	-
12	Ec 1mm	Mv 2 mm	> 100	-	95.3	> 10000	> 10000
13	n.a.	Mv 2 mm	> 100	-	> 100	5095	3651
14	n.a.	Mv 2 mm	-	-	> 100	-	-
15	Bm 2mm	Mv 3 mm	72.7	-	> 100	2904	4244
16	-	-	58.0	-	> 100	-	-
17	n.a.	Mv 3 mm	> 100	-	89.6	> 10000	> 10000
18	n.a.	Mv 2 mm	> 100	-	94.1	> 10000	> 10000
19	n.a.	Mv 3 mm	> 100	-	97.2	6945	9261
20	Bm 2mm	Mv 1 mm	44.2	-	> 100	1412	3303
21	n.a.	Mv 5 mm	0.0	48.1	70.2	2611	3411
Chloroquine	-	-	-	-	-	48	3.2

na = not active; ti = total inhibition zone in mm, no colonies of the test organism were growing within the inhibition zone; gi = growth inhibition zone in mm, some colonies of the test organism were growing within the inhibition zone. Inhibition zones were measured from the edge of the filter discs.

[a] Tested against *Bacillus megaterium* (Bm) and *Escherichia coli* (Ec), benzylpenicillin and streptomycin sulphate were positive controls: inhibition zones of benzylpenicillin 2 mm (Ec), 17 mm (Bm); inhibition zones of streptomycin sulphate 2 mm (Ec), 10 mm (Bm). Test concentration: 50 μg/disc.

[b] Tested against *Eurotium repens* (Er), *Fusarium oxysporum* (Fo), *Microbotryum violacea* (Mv), and *Mycotypha microspora* (Mm), miconazol was positive control: inhibition zone 25 mm (Mv), 25 mm (Er), 3 mm (Fo), and 7 mm (Mm), respectively. Test concentration: 50 μg/disc.

[c] Values give % residual tyrosine kinase (TK $p56^{lck}$) activity. Lck = lymphocytic kinase.

[d] Values give % residual reverse transcriptase (HIV-1) activity.

[e] Antiplasmodial activity was measured against two reference strains of *Plasmodium falciparum* K1 (Thailand; resistant to chloroquine and pyrimethamine), and NF 54 (an airport strain of unknown origin; susceptible to standard antimalarials).

Drechslerine A (**6**) was obtained as an amorphous white powder (0.7 mg/L). $[\alpha]^{20}_{D}$ = - 2.0° (*c* = 0.10, EtOH); compound was not UV active in the range of 200-400 nm; IR (film) ν_{max} 3580, 3270, 2920, 2315 cm^{-1}; ^{1}H and ^{13}C NMR data see Tables 13 and 14; FABMS *m/z* 275 $[M+Na]^{+}$; EIMS *m/z* 234 $[M-H_2O]^{+}$ (22), 219 (18), 188 (42), 173 (62), 145 (100), 133 (48), 119 (54), 105 (68), 91 (62); HREIMS *m/z* 234.1614 (calcd. for $C_{15}H_{22}O_2$ $[M-H_2O]^{+}$ 234.1614).

Drechslerine B (**7**) was obtained as white needles (1.2 mg/L): Mp. 125.0°C, $[\alpha]^{20}_{D}$ = - 2.0° (*c* = 0.10, EtOH); compound was not UV active in the range of 200-400 nm; IR (film) ν_{max} 3270, 2950, 1455 cm^{-1}; ^{1}H and ^{13}C NMR data see Tables 13 and 14; EIMS *m/z* 224 $[M]^{+}$ (10), 206 (42), 193 (38), 181 (24), 175 (68), 163 (24), 139 (72), 133 (100), 119 (42), 105 (74); HREIMS *m/z* 206.1664 (calcd. for $C_{14}H_{22}O$ $[M-H_2O]^{+}$ 206.1665).

Drechslerine C (**8**) was obtained as a colourless oil (0.4 mg/L). $[\alpha]^{20}_{D}$ = -3.2° (*c* = 0.10, EtOH); UV (EtOH) λ_{max} (log ε) 235 (3.63); IR (film) ν_{max} 3420, 2925, 2870, 1745 cm^{-1}; ^{1}H and ^{13}C NMR data see Tables 13 and 14; EIMS *m/z* 250 $[M]^{+}$ (22), 232 (28), 207 (60), 189 (100), 177 (58), 164 (52), 150 (76), 133 (38), 119 (58), 105 (52); HREIMS *m/z* 250.1563 (calcd. for $C_{15}H_{22}O_3$ 250.1563).

Helminthosporol (**9**) was obtained as a colourless oil (9.3 mg/L). $[\alpha]^{20}_{D}$ = -24.0° (*c* = 0.07, $CHCl_3$); Lit. (Tamura *et al.*, 1963) $[\alpha]^{20}_{D}$ = -28.7° (*c* = 1.93, $CHCl_3$); UV (EtOH) λ_{max} (log ε) 263 (3.72); Lit. (Tamura *et al.*, 1963) UV (EtOH) λ_{max} (ε) 267 (9700); IR (film) ν_{max} 3420, 2920, 1650, 1470 cm^{-1}; Lit. (Tamura *et al.*, 1963) IR (nujol) ν_{max} 3440, 1645, 1610 cm^{-1}. ^{1}H and ^{13}C NMR data see Tables 13 and 14; Lit. (Pena-Rodriguez *et al.*, 1988) ^{1}H NMR data (250 MHz, $CDCl_3$) ppm 10.03 (s), 3.63 (dd, *J* = 11.5, 5.5 Hz), 3.32 (dd, *J* = 11.0, 8.0 Hz), 2.00 (s), 1.66 (dd, *J* = 8.0, 5.0 Hz), 1.05 (d, *J* = 6.0 Hz), 1.02 (s), 0.75 (d, *J* = 5.0 Hz). ^{13}C NMR data were not available from literature. EIMS *m/z* 236 $[M]^{+}$ (72), 218 (38), 205 (34), 189 (26), 175 (24), 154 (30), 147 (34), 133 (46), 123 (65), 107 (86), 91 (100); HREIMS *m/z* 236.1770 (calcd. for $C_{15}H_{24}O_2$ 236.1770).

9-Hydroxyhelminthosporol (**10**) was obtained as an amorphous white powder (0.5 mg/L). $[\alpha]^{20}_D$ = +39.5° (*c* = 0.40, EtOH); UV (EtOH) λ_{max} (log ε) 268 (3.66); IR (film) ν_{max} 3415, 2935, 1730, 1640 cm^{-1}; ^1H and ^{13}C NMR data see Tables 13 and 14; EIMS *m/z* 252 $[M]^+$ (2), 194 (26), 176 (100), 161 (50), 147 (48), 135 (62), 119 (32), 107 (53), 91 (53); HREIMS was not performed due to the low intensity of the molecular ion.

Drechslerone A (**11**) was obtained as an amorphous white powder (0.4 mg/L). $[\alpha]^{20}_D$ = -10.5° (*c* = 0.15, EtOH); UV (EtOH) λ_{max} (log ε) 220 (3.08); IR (film) ν_{max} 2950, 2920, 2865, 1730, 1685, 1460, 1030 cm^{-1}; ^1H and ^{13}C NMR data see Tables 13 and 14; EIMS *m/z* 224 $[M]^+$ (65), 209 (2), 193 (28), 181 (22), 175 (8), 163 (4), 153 (15), 140 (100), 123 (27), 107 (38); HREIMS *m/z* 224.1770 (calcd. for $C_{14}H_{24}O_2$ 224.1770).

Drechslerone B (**12**) was obtained as a colourless oil (0.5 mg/L). $[\alpha]^{20}_D$ = -71.1° (*c* = 0.40, EtOH); UV (EtOH) λ_{max} (log ε) 217 (3.10); IR (film) ν_{max} 3455, 2930, 1730, 1455 cm^{-1}; ^1H and ^{13}C NMR data see Tables 13 and 14; EIMS *m/z* 250 $[M]^+$ (4), 235 (8), 217 (4), 192 (78), 173 (32), 147 (20), 125 (100), 105 (42), 91 (38); HREIMS *m/z* 250.1563 (calcd. for $C_{15}H_{22}O_3$ 250.1563).

Drechslerine D (**13**) was obtained as an amorphous white powder (1.1 mg/L). $[\alpha]^{20}_D$ = +9.5° (*c* = 0.40, EtOH); UV (EtOH) λ_{max} (log ε) 264 (2.43); IR (film) ν_{max} 3450, 2960, 2870, 1725, 1655 cm^{-1}; ^1H and ^{13}C NMR data see Tables 13 and 14; EIMS *m/z* 266 $[M]^+$ (12), 252 (4), 235 (18), 219 (12), 191 (18), 188 (32), 173 (47), 163 (26), 148 (70), 145 (46), 119 (82), 107 (57), 91 (42); HREIMS *m/z* 266.1874 (calcd. for $C_{16}H_{26}O_3$ 266.1875).

Drechslepoxide A (**14**) was obtained as an amorphous white powder (0.2 mg/L). $[\alpha]^{20}_D$ = -28.1° (*c* = 0.20, EtOH); UV (EtOH) λ_{max} (log ε) 226 (3.14); IR (film) ν_{max} 2960, 2930, 2875, 2855, 1735, 1460 cm^{-1}; ^1H and ^{13}C NMR data see Tables 13 and 14; EIMS *m/z* 250 $[M]^+$ (18), 235 (6), 221 (3), 207 (18), 191 (5), 177 (24), 163 (15), 151 (32), 137 (62), 121 (47), 107 (100), 91 (95); HREIMS *m/z* 250.1563 (calcd. for $C_{15}H_{22}O_3$ 250.1563).

Drechsleral (**15**) was obtained as a yellowish oil (1.2 mg/L). $[\alpha]^{20}_{D}$ = -26.9° (*c* = 0.47, EtOH); UV (EtOH) λ_{max} (log ε) 264 (2.98); IR (film) ν_{max} 3430, 2935, 2875, 1715 cm^{-1}; ^{1}H and ^{13}C NMR data see Tables 13 and 14; EIMS *m/z* 252 $[M]^{+}$ (8), 232 (70), 224 (18), 206 (100), 193 (42), 175 (56), 163 (32), 139 (68), 107 (82), 91 (74); HREIMS *m/z* 252.1719 (calcd. for $C_{15}H_{24}O_3$ 252.1719).

cis-Sativenediol (**16**) was obtained as a clear oil (0.5 mg/L). $[\alpha]^{20}_{D}$ = -83.5° (*c* = 0.13, EtOH); Lit. (Nukina *et al.*, 1975) $[\alpha]^{25}_{D}$ = -119° (*c* = 0.94, $CHCl_3$); UV (EtOH) λ_{max} (log ε) 227(sh) (3.47); IR (film) ν_{max} 3610, 3380, 2950, 2930, 2870, 2840 cm^{-1}; Lit. (Nukina *et al.*, 1975) IR (CCl_4) ν_{max} 3670, 3360 cm^{-1} ; ^{1}H and ^{13}C NMR data see Tables 13 and 14; Lit. (Nukina *et al.*, 1975) ^{1}H NMR data ($CDCl_3$) 0.89 (d, *J* = 7.0 Hz), 0.95 (d, *J* = 7.0 Hz), 1.08 (s), 3.68 (d, *J* = 6.0 Hz), 4.08 (d, *J* = 6.0 Hz), 4.66 (d), 4.98 (d); EIMS *m/z* 236 $[M]^{+}$ (12), 207 (40), 189 (15), 177 (86), 157 (15), 147 (38), 137 (50), 121 (75), 107 (100); Lit. (Nukina *et al.*, 1975) *m/z* 236 $[M]^{+}$; HREIMS *m/z* 236.1770 (calcd. for $C_{15}H_{24}O_2$ 236.1770).

Drechslepoxide B (**17**) was obtained as an amorphous white powder (0.2 mg/L). $[\alpha]^{20}_{D}$ = -11.5° (*c* = 0.26, EtOH); compound was not UV active in the range of 200-400 nm; IR (film) ν_{max} 3550, 2925, 2870, 1650 cm^{-1}; ^{1}H and ^{13}C NMR data see Tables 13 and 14; EIMS *m/z* 252 $[M]^{+}$ (44), 234 (6), 221 (15), 209 (12), 193 (34), 180 (32), 161 (54), 149 (46), 137 (44), 121 (46), 105 (66), 91 (100); HREIMS *m/z* 252.1719 (calcd. for $C_{15}H_{24}O_3$ 252.1719).

Drechslerine E [(+)-secolongifolene diol] (**18**) was obtained as an amorphous white powder (0.5 mg/L). $[\alpha]^{20}_{D}$ = +37.9° (*c* = 0.08, EtOH); Lit. (Yadav *et al.*, 1983) $[\alpha]^{20}_{D}$ = -23 ±2° (*c* = 1.06, $CHCl_3$) for (-)-secolongifolene diol; compound was not UV active in the range of 200-400 nm; IR (film) ν_{max} 3330, 2925, 1730, 1460 cm^{-1}; ^{1}H and ^{13}C NMR data see Tables 13 and 14; EIMS *m/z* 238 $[M]^{+}$ (20), 220 (40), 205 (56), 193 (28), 189 (57), 175 (64), 153 (38), 138 (82), 133 (59), 119 (72), 107 (100), 91 (74); HREIMS *m/z* 220.1821 (calcd. for $C_{15}H_{24}O$ $[M\text{-}H_2O]^{+}$ 220.1821).

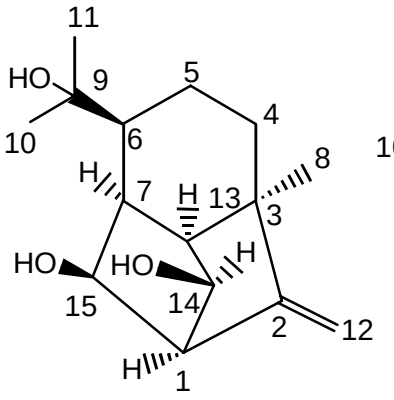
Isocochlioquinone A (**19**) was obtained as yellow pigments (0.7 mg/L). $[\alpha]^{20}_{D}$ = +117.3° (*c* = 0.21, EtOH); Lit. (Lim *et al.*, 1998) $[\alpha]^{22}_{D}$ = +130.9° (*c* = 0.1, EtOH); UV (EtOH) λ_{max} (log ε) 214 (4.16), 338 (3.59), 283 (3.97); Lit. (Miyagawa *et al.*, 1994) UV (EtOH) λ_{max} (ε) 243 (12000), 283 (24000), 383 (6500); IR (film) ν_{max} 3360, 3350, 2920, 2850 cm^{-1}; Lit. (Miyagawa *et al.*, 1994) IR (KBr) ν_{max} 3430, 1730, 1642, 1580 cm^{-1}; ^{1}H NMR data (300 MHz, $CDCl_3$) 0.82 (t, *J* = 7.5 Hz), 0.86 (d, *J* = 6.8 Hz), 1.05 (m), 1.12 (s), 1.17 (s), 1.17 (d, *J* = 7.0 Hz), 1.17 (m), 1.27 (m), 1.43 (m), 1.45 (m), 1.46 (s), 1.51 (m), 1.61 (m), 1.72 (m), 1.80 (m), 2.03 (s), 2.04 (m), 2.73 (dd, *J* = 3.0, 12.8 Hz), 2.77 (s), 3.14 (dd, *J* = 12.0, 3.6 Hz), 3.25 (dd, *J* = 12.0, 3.0 Hz), 3.55 (dq, *J* = 7.2, 1.5 Hz), 5.03 (dd, *J* = 7.5, 5.3 Hz), 6.50 (s); Lit. (Miyagawa *et al.*, 1994) ^{1}H NMR data (400 MHz, $CDCl_3$) 0.89 (t, *J* = 7.0 Hz), 0.88 (d, *J* = 6.8 Hz), 1.13 (s), 1.19 (s), 1.19 (s), 1.20 (d, *J* = 7.0), 1.25 (+), 1.30 (+), 2.75 (+), 1.46 (s), 1.60 (+), 1.60 (+), 1.63 (+), 1.80 (+), 2.06 (+), 2.77 (s), 2.58 (brs), 3.16 (dd, *J* = 12.0, 3.8 Hz), 3.27 (dd, *J* = 12.5, 2.5 Hz), 3.46 q (*J* = 7.0 Hz), 5.18 (dd, *J* = 7.5, 5.0 Hz), 5.22, 6.40 (s), 10.76; ^{13}C NMR data (300 MHz, $CDCl_3$) 198.3 (s), 170.8 (s), 153.1 (s), 143.8 (s), 139.7 (s), 135.0 (s), 108.2 (d), 106.9 (s), 85.3 (d), 83.7 (d), 83.5 (s), 80.5 (d), 71.9 (s), 60.4 (d), 37.6 (t), 37.3 (t), 36.4 (d), 34.3 (d), 26.0 (q), 24.9 (t), 24.2 (t), 23.7 (q), 22.0 (q), 21.3 (t), 20.9 (q), 18.0 (q), 15.3 (q), 12.3 (q), 11.1 (q); Lit. (Miyagawa *et al.*, 1994) ^{13}C NMR data ($CDCl_3$) 198.5 (s), 170.5 (s), 153.2 (s), 144.0 (s), 140.2 (s), 135.3 (s), 108.2 (d), 107.0 (s), 85.5 (d), 83.6 (d), 83.3 (s), 79.0 (d), 72.0 (s), 60.5 (d), 37.6 (t), 37.3 (t), 36.2 (d), 35.6 (s), 35.5 (d), 26.5 (t), 26.0 (q), 25.0 (t), 23.8 (q), 22.0 (q), 21.3 (t), 20.8 (q), 21.3 (t), 17.5 (q), 13.5 (q), 12.5 (q), 11.5 (q); EIMS *m/z* 532 $[M]^{+}$ (0.1), 457 (0.9), 433 (0.4), 401 (0.8), 390 (0.4), 369 (0.9), 281 (68), 264 (24), 238 (14), 216 (10), 184 (18), 159 (18), 128 (28), 126 (40), 112 (28); Lit. (Miyagawa *et al.*, 1994) EIMS *m/z* 532 $[M]^{+}$ (38), 472 (48), 457 (100), 249 (30), 179 (42); HREIMS *m/z* 532.3024 (calcd. for $C_{30}H_{44}O_8$ 532.3024).

Isocochlioquinone C (**20**) was obtained as yellow pigments (1.6 mg/L). $[\alpha]^{20}_{D}$ = +123.3° (*c* = 0.20, EtOH); Lit. (Lim *et al.*, 1998) $[\alpha]^{22}_{D}$ = +287.4° (*c* = 0.27, MeOH); UV (EtOH) λ_{max} (log ε) 211 (4.06), sh 238 (3.71), 281 (3.84), sh 290 (3.78), 382 (3.39); Lit. (Lim *et al.*, 1998) UV (MeOH) λ_{max} (ε) 250 (8600), 302 (5500); IR (film) ν_{max} 2960, 2930, 1735, 1715, 1455 cm^{-1}; Lit. (Lim *et al.*, 1998) IR (film) ν_{max} 3420, 2980, 2950, 1710, 1665, 1580, 1395, 1238, 1100 cm^{-1}, ^{1}H NMR data (300 MHz,

$CDCl_3$) 0.70 (t, *J* = 7.5 Hz), 1.05 (d, *J* = 6.8 Hz), 1.11 (s), 1.11 (s), 1.17 (s), 1.17 (m), 1.27 (m), 1.30 (m), 1.35 (d, *J* = 7.0 Hz), 1.47 (s), 1.61 (m), 1.71 (m), 1.80 (m), 2.03 (m), 2.07 (m), 2.51 (brq, *J* = 6.8 Hz), 2.73 (m), 2.77 (s), 3.14 (dd, *J* = 3.6, 12.0 Hz), 3.25 (dd, *J* = 3.0, 11.7 Hz), 4.28 (q, *J* = 7.0 Hz), 6.25 (s); Lit. (Lim *et al.*, 1998) ^{1}H NMR data ($CDCl_3$) 0.85 (t, *J* = 7.4 Hz), 0.93 (d, *J* = 6.7 Hz), 1.11 (s), 1.17 (s), 1.18 (s), 1.33 (d, *J* = 7.0), 1.47 (s), 2.08 (m), 2.56 (m), 2.73 (+), 2.78 (s), 3.13 (dd, *J* = 12.0, 3.7 Hz), 3.22 (dd, *J* = 12.0, 2.7), 4.24 (q, *J* = 7.0), 5.35 (OH), 6.24 (s), 10.78 (OH); ^{13}C NMR data (300 MHz, $CDCl_3$) 213.7 (s), 198.3 (s), 153.7 (s), 144.3 (s), 136.3 (s), 134.8 (s), 107.4 (s), 107.4 (d), 85.3 (d), 83.6 (s), 83.6 (d), 71.9 (s), 60.4 (d), 46.5 (d), 44.7 (d), 37.6 (t), 37.3 (t), 35.4 (s), 26.0 (q), 25.4 (t), 24.9 (t), 23.7 (q), 22.0 (q), 21.2 (t), 16.9 (q), 16.0 (q), 12.3 (q), 11.6 (q); Lit. (Lim *et al.*, 1998) ^{13}C NMR data ($CDCl_3$) 214.0 (s), 198.3 (s), 153.7 (s), 144.3 (s), 136.5 (s), 134.7 (s), 107.3 (s), 107.3 (d), 85.3 (d), 83.6 (s), 83.6 (d), 71.9 (s), 60.4 (d), 46.3 (d), 45.5 (d), 37.6 (t), 37.3 (t), 35.4 (s), 26.6 (q), 26.0 (t), 24.9 (t), 23.7 (q), 22.0 (q), 21.3 (t), 16.0 (q), 15.8 (q), 12.3 (q), 11.6 (q); EIMS *m/z* 488 $[M]^+$ (52), 473 (94), 455 (22), 404 (24), 266 (100), 232 (78), 179 (28), 149 (56), 125 (42), 111 (56); HREIMS *m/z* 488.2763 (calcd. for $C_{28}H_{40}O_7$ 488.2763). Lit. (Lim *et al.*, 1998) EIMS *m/z* 488 $[M]^+$ (57), 473 (100), 429 (8), 404 (25), 386 (6), 343 (5), 305 (5), 265 (15), 247 (5), 223 (8), 179 (57), 152 (5), 121 (11), 85 (30), 57 (84).

Cochlioquinone B (**21**) was obtained as yellow pigments (0.9 mg/L). $[\alpha]^{20}_D$ = +111.5° (*c* = 0.10, EtOH); Lit. (Lim *et al.*, 1998) $[\alpha]^{22}_D$ = +108.4° (*c* = 0.1, EtOH); UV (EtOH) λ_{max} (log ε) 262 (3.88); Lit. (Miyagawa *et al.*, 1994) UV (EtOH) λ_{max} (ε) 260 (22000); IR (film) ν_{max} 2960, 2920, 2850, 1730 cm^{-1}; Lit. (Miyagawa *et al.*, 1994) IR (KBr) ν_{max} 3450, 1715, 1678, 1650, 1605 cm^{-1}; ^{1}H NMR data (300 MHz, $CDCl_3$) 0.88 (t, *J* = 7.5 Hz), 0.88 (s), 1.09 (d, *J* = 7.3 Hz), 1.16 (s), 1.18 (s), 1.22 (m), 1.26 (s), 1.30 (d, *J* = 7.1 Hz), 1.39 (m), 1.45 (m), 1.48 (m), 1.55 (m), 1.65 (m), 1.78 (m), 1.85 (m), 1.88 (m), 2.11 (m), 2.17 (m), 2.52 (dd, *J* = 18.3, 4.9 Hz), 2.68 (dq, *J* = 7.1, 1.1 Hz), 3.11 (dd, *J* = 11.7, 3.4 Hz), 3.21 (dd, *J* = 11.7, 3.0 Hz), 4.1 (dq, *J* = 7.3, 1.1 Hz), 6.49 (brd, *J* = 1.1 Hz); Lit. (Miyagawa *et al.*, 1994) ^{1}H NMR data (400 MHz, $CDCl_3$) 0.87 (t, *J* = 7.2 Hz), 0.89 (s), 1.15 (d, *J* = 7.0 Hz), 1.17 (s), 1.19 (s), 1.20 (+), 1.28 (s), 1.29 (d, *J* = 7.0 Hz), 1.39 (qnt, *J* = 7.2 Hz), 1.46 (dd, *J* = 12.4, 4.6 Hz), 1.50 (+), 1.63 (+), 1.80 (+), 1.90 (m), 2.14 (dd, *J* = 18.7, 12.4 Hz), 2.18 (m), 2.50 (+), 2.54 (dd, *J* = 18.7, 4.6

Hz), 2.70 (sxt, *J* = 7.2 Hz), 3.22 (dd, *J* = 12.0, 3.6 Hz), 3.33 (dd, *J* = 12.0, 2.9 Hz), 4.10 (dq, *J* = 7.0, 1.1 Hz), 6.50 (d, *J* = 1.1 Hz); ^{13}C NMR data (300 MHz, $CDCl_3$) 212.7 (s), 186.7 (s), 181.4 (s), 152.2 (s), 145.4 (s), 133.4 (d), 118.5 (s), 84.9 (d), 84.0 (d), 80.7 (s), 71.8 (s), 47.0 (d), 43.0 (d), 37.2 (t), 36.8 (t), 35.5 (s), 27.1 (q), 25.9 (t), 25.2 (t), 23.8 (q), 21.3 (t), 20.8 (q), 16.5 (t), 15.9 (q), 15.2 (q), 12.2 (q), 11.6 (q); Lit. (Miyagawa *et al.*, 1994) ^{13}C NMR data ($CDCl_3$) 212.6 (s), 186.6 (s), 181.5 (s), 152.2 (s), 145.5 (s), 133.5 (d), 118.6 (s), 85.0 (d), 84.1 (d), 80.7 (s), 71.9 (s), 47.4 (d), 46.6 (d), 43.0 (d), 37.3 (t), 36.9 (t), 35.6 (s), 26.1 (q), 25.8 (t), 25.2 (t), 23.9 (q), 21.4 (t), 16.5 (t), 16.5 (q), 14.9 (q), 12.2 (q), 11.7 (q); EIMS *m/z* 472 $[M]^+$ (0.1), 432 (0.7), 418 (3.3), 361 (1.3), 307 (4), 293 (56), 281 (24), 264 (8), 238 (6), 167 (16), 149 (100), 127 (24). Lit. (Miyagawa *et al.*, 1994) EIMS *m/z* 472 $[M]^+$ (9), 388 (93), 370 (26), 243 (20), 223 (35), 205 (22), 165 (33), 85 (52), 57 (100).



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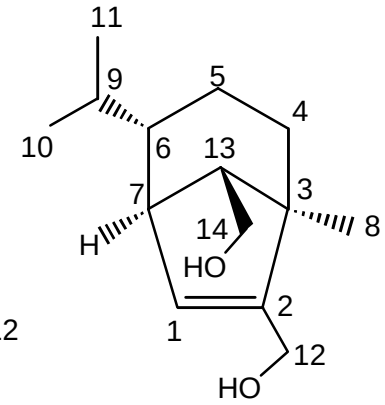

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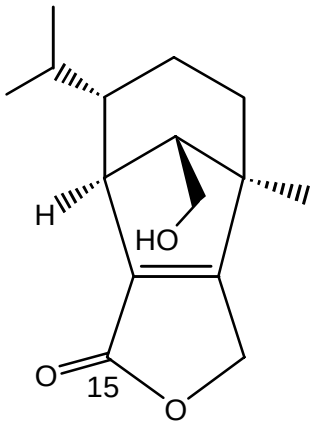

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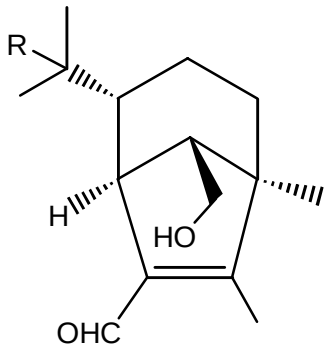
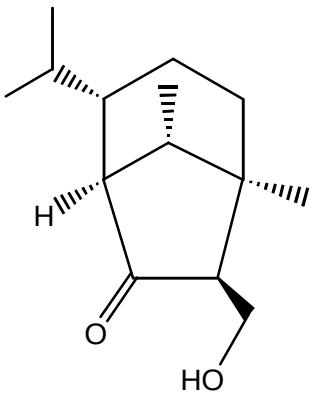


9 R = H
10 R = OH



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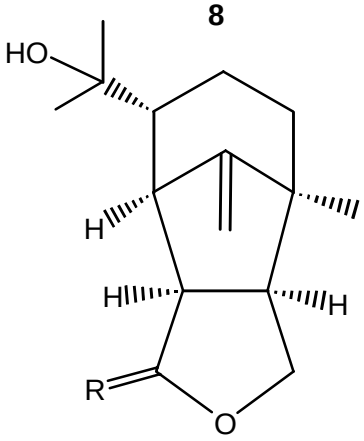
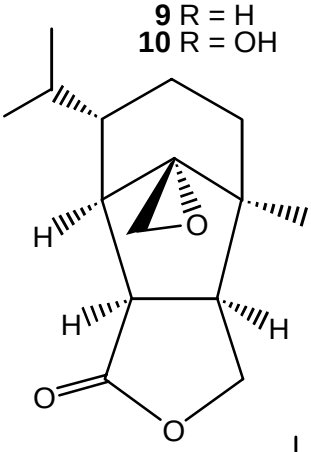


12 R = O
13 R = β H , α OCH_3



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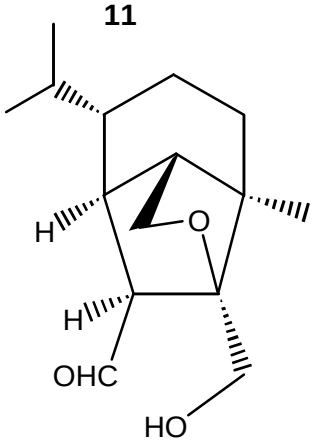

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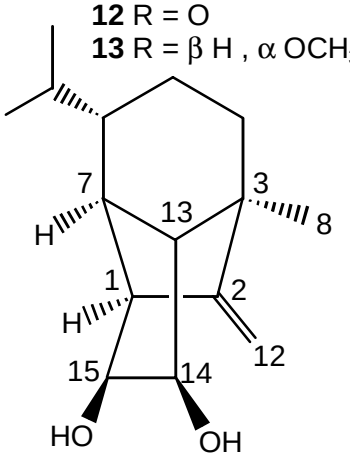

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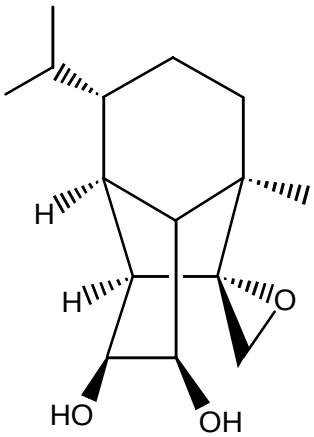

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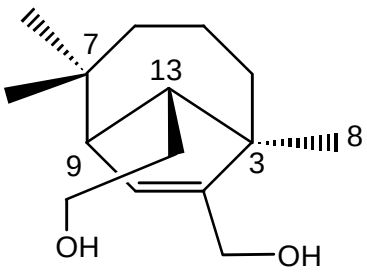

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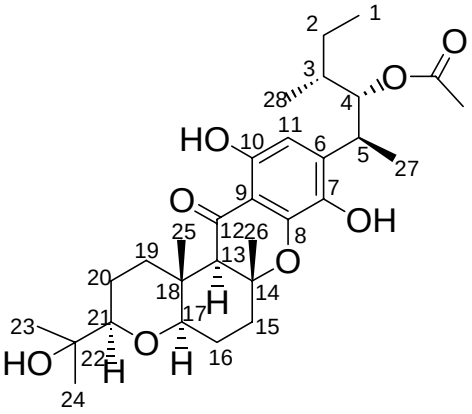
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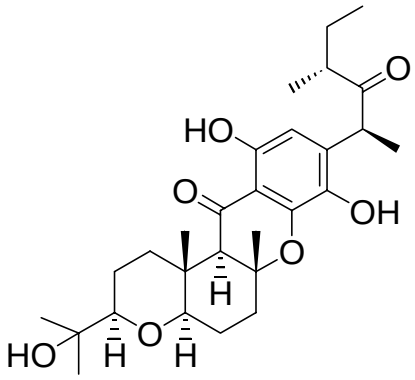
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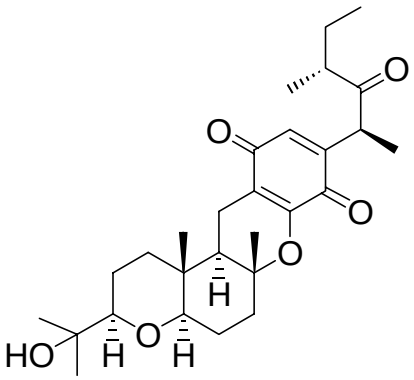
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4.5 5-Hydroxyramulosin, a new natural product from *Phoma tropica*, a marine-derived fungus from the alga *Fucus spiralis*

Abstract

The fungus *Phoma tropica* was isolated from the inner tissue of the marine brown alga *Fucus spiralis*. After large-scale cultivation the fungus was investigated for its secondary metabolite content. The new compound 5-hydroxyramulosin (**22**) was isolated together with the known natural product 7-methoxycoumarin (**23**). Structures were elucidated using spectroscopic methods, mainly 1D and 2D NMR as well as X-ray analysis.

Introduction

The fungal genus *Phoma* is known for its diverse spectrum of secondary metabolites which is reflected by numerous studies, e.g., the antibiotic phomalide which was produced by *P. lingam* (Pedras *et al.*, 1993), cytochalasin T separated from *P. exigua* var. *heteromorpha* (Capasso *et al.*, 1991), and the compound betaenone D isolated from *P. betae* (Ichihara *et al.*, 1983). The Chapmann & Hall database shows 65 secondary metabolite entries for this genus (Buckingham (ed.), 1996). A chemotaxonomic investigation showed that marine-derived *Phoma* spp., differ significantly from their terrestrial counterparts with respect to their secondary metabolite content (Osterhage *et al.*, 2000). Based on these data an investigation into the secondary metabolite content of *P. tropica* was undertaken.

During our investigations dealing with the isolation, cultivation and secondary metabolite production of marine-derived fungi *P. tropica* was obtained from the marine brown alga *Fucus spiralis*. Algal material was surface sterilised with EtOH 70 % and sodium hypochlorite solution (2 %) to ensure fungi were isolated from the inner algal tissue and thus to increase the possibility that the isolate is a fungal endophyte. The EtOAc extract of a culture of this fungus was found to exhibit antimicrobial activity. Thus, the fungus was further investigated in order to identify the natural products responsible for this activity.

Materials and methods

Isolation and Taxonomy of the fungus

The algal material was collected by snorkelling around the Azores, Atlantic Ocean, Portugal. The inner tissue was cut into small pieces and placed on agar plates containing isolation medium (15 g/L agar and 1000 ml sea water from the sample collecting site. After autoclaving the antibiotics benzylpenicillin and streptomycin sulphate were added by sterile filtration). Fungal colonies growing out of the alga were transferred to medium for sporulation (1.0 g glucose, 0.1 g yeast extract, 0.5 g peptone from meat, enzymatic digest, 15 g agar, and 1000 ml sea water, pH 8) in order to enable taxonomy of the isolates.

Cultivation

The microorganism was cultured at 20 °C for 4 weeks in 14 litres of liquid malt extract Soya meal agar (30 g malt extract, Merck, 3 g peptone from Soya meal, papain-digest, Merck, 1000 mL ASW).

Biological activity, see chapter 3.5.

Extraction and Isolation

Prior to extraction with 42 L EtOAc the fungal culture was blended using the Ultra Turrax model T 25 at 8000 min^{-1}. The resultant EtOAc extract (3.9 g) was purified employing a combination of chromatographic techniques. First, it was passed over normal phase silica (vacuum liquid chromatography, VLC) using a gradient of petroleum ether (PE)/EtOAc/MeOH as eluent to yield 28 fractions each of 250 mL. VLC fractions 7-12 in which antimicrobial activity was concentrated were combined and separated by VLC again yielding 14 fractions. Fractions 7 and 8 of the second VLC were combined and separated by normal phase HPLC (eluted with PE/acetone 80:20) and further purified by RP-18 HPLC yielding compound **22** (26 mg, eluted with methanol/H_2O 60:40). Fractions 5 and 6 of the second VLC were combined because of similar ^1H NMR information and separated by normal phase HPLC yielding compound **23** (20 mg, eluted with PE/acetone 80:20).

Results and Discussion

In the present study the isolation and structure elucidation of 5-hydroxyramulosin (**22**) and 7-methoxycoumarin (**23**) obtained from *P. tropica* is described. The fungus was cultivated in 14 L of a liquid malt extract Soya meal medium with added artificial sea salt. Successive fractionation of the EtOAc extract by vacuum-liquid chromatography (VLC), and normal (Si-60) and reversed (RP-18) phase HPLC yielded compounds **22** and **23**.

5-Hydroxyramulosin (**22**) has the molecular formula $C_{10}H_{14}O_4$ as deduced by accurate mass measurement. Its ^{13}C NMR spectrum contained 10 signals (see Table 16). From the ^{13}C NMR spectroscopic data (^{1}H decoupled and DEPT) and from the IR spectrum it was evident that one of the four elements of unsaturation implied by the molecular formula of **22**, could be attributed to a carbon-carbon double bond [δ 92.1 (C-8a, s), 172.3 (C-8, s)], and one to a lactone carbonyl [δ 174.2 (C-1, s)] as the only multiple bonds within the molecule; the molecule is thus bicyclic. These data also showed the two other oxygen atoms present in the molecule to be there in the form of hydroxyl groups. The ^{1}H and ^{13}C NMR spectra further revealed the presence of a methyl group [δ 1.39 (H_3-9, d)], three methylene groups [δ 24.2 (C-7, t); 27.9 (C-6, t); 32.7 (C-4, t)], three methine groups [δ 37.4 (C-4a, d)], two of them being attached to oxygen [δ 66.0 (C-5, d); 76.1 (C-3, d)]. From the ^{1}H-^{1}H COSY spectrum couplings were observed between H_3-9 and H-3, between H-3 and H_2-4, between H_2-4 and H-4a, between H-4a and H-5, between H-5 and H_2-6, and between H_2-6 and H_2-7, showing C-9 to be bonded to C-3, C-3 to C-4, C-4 to C-4a, C-4a to C-5, C-5 to C-6, and C-6 to C-7. This information together with the data obtained from a ^{1}H-^{13}C HMBC spectrum of **22** allowed the planar structure of **22** to be deduced. Thus, diagnostic HMBC correlations from the resonance for the proton of the hydroxyl group at C-8 to those for C-7, C-8a, C-8 and C-1 showed the hydroxyl group to be attached to C-8 which is further bonded to C-7 and C-8a. Diagnostic HMBC correlations between the resonance for H-5 and that for C-8a, and between the resonances for H_2-4 and that for C-8a showed C-4a to be bonded to C-8a and thus completed the first ring within **22**. By deduction the lactone function had to reside between C-3 and C-8a, thus completing the planar structure of **22**. As **22** was crystalline a single crystal X-ray analysis of it was performed and the relative configuration determined to be as shown in Fig. 9.

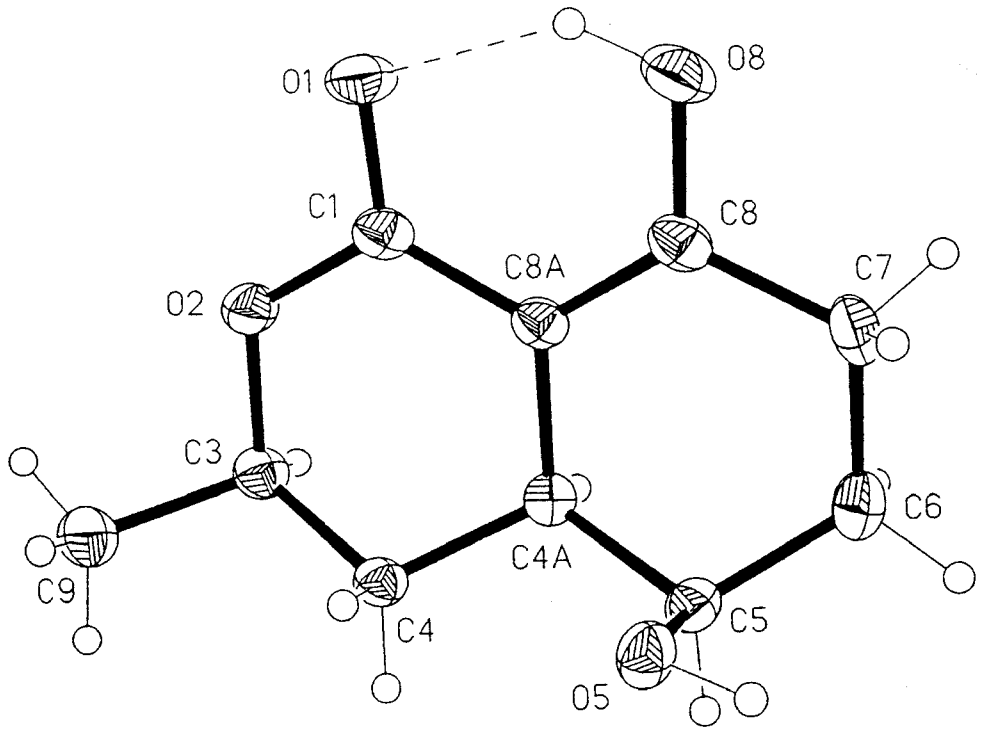


Figure 9. Results of the single X-ray analysis of 5-hydroxyramulosin (**22**). Ellipsoids represent 50 % probability levels. H atom radii are arbitrary.

Compound **22** is a new natural product. The analogous aromatic compound 5-hydroxymellein is a common fungal secondary metabolite and was isolated from the culture broth of the phytopathogenous fungus *Septoria nodorum* Berk (Devys *et al.*, 1994) as well as from the apple pathogen *Botryosphaeria obtusa* (Venkatasubbaiah & Chilton, 1990).

Table 16. ^{1}H ($CDCl_3$, 300 MHz) and ^{13}C NMR ($CDCl_3$, 75 MHz) data[a] for 5-hydroxyramulosin (**22**).

position	δ C	type[b]	δ H
1	174.2	s	-
3	76.1	d	4.46 (ddq, *J* = 2.4, 6.4, 11.3 Hz)
4	32.7	t	1.70 (m)
			1.85 (m)
4a	37.4	d	2.67 (m)
5	66.0	d	4.03 (m)
6	27.9	t	2.2 (dddd, *J* = 1.3, 4.1, 7.2, 14.4 Hz)
			1.82 (m)
7	24.2	t	2.57 (ddd, *J* = 2.3, 7.2, 12.2 Hz)
			2.35 (m)
8	172.3	s	-
8a	92.1	s	-
9	21.8	q	1.39 (d, *J* = 6.4 Hz)

[a] All assignments are based on extensive 1 D and 2 D NMR experiments.

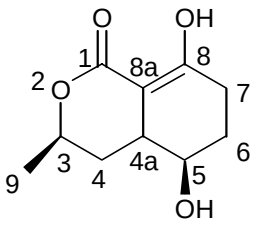
[b] Attached protons as determined by DEPT (C = s, CH = d, CH_2 = t, CH_3 = q).

Compound **23** was identified as 7-methoxycoumarin (herniarin) by comparison of its ^{1}H and ^{13}C NMR data with published values (Zubia *et al.*, 1992; Osborne, 1989). This compound was first isolated from the leaves of *Herniaria hirsuta* in 1889. Compounds like **23** are known to occur commonly in higher plants, e.g., *Matricaria* spp. (first report in 1914) and *Lavandula* sp. (1927). Production of herniarin by microorganisms has not been reported.

Both compounds were subjected to different assays investigating their potential for pharmaceutical use. While **22** did not show any activity in the applied assay systems, **23** reduced the enzyme activity of HIV-1 RT to 86 % at a concentration of 66 µg/mL.

5-Hydroxyramulosin (**22**) was obtained as white crystals (1.9 mg/L). Mp. 191°C, $[\alpha]^{20}_{D}$ = +17.12° (*c* = 0.20, EtOH); UV (EtOH) λ_{max} (log ε) 264 nm (3.62); IR (film) ν_{max} 3450, 2980, 2910, 1625, 1590 cm^{-1}; 1H and ^{13}C NMR data see Table 16; EIMS *m/z* 198 [M^+] (40), 180 (26), 154 (100), 136 (92), 121 (50), 111 (19), 95 (19), 85 (16), 68 (49), 55 (33), 43 (32); HREIMS *m/z* 198.0887 (calcd. for $C_{10}H_{14}O_4$ 198.0888).

7-Methoxycoumarin (= herniarin) (**23**) was obtained as white amorphs (1.4 mg/L). Mp. 114°C, Lit. (Zubia *et al.*, 1992) 115-116 °C. UV (EtOH) λ_{max} (log ε) 274 (3.93), 338 (3.55); Lit. (Mangini & Passerini, 1957) UV (EtOH) λ_{max} (log ε) 283 (4.03), 313 (3.92) IR (film) ν_{max} 1705, 1610, 1570, 1390, 1285 cm^{-1}; Lit. (Zubia *et al.*, 1992) IR (film) ν_{max} 1701, 1608, 1395, 1348, 1280, 1122, 1022 cm^{-1}. ^1H NMR data (300 MHz, $CDCl_3$) ppm 7.65 (d, *J* = 9.6 Hz, H-4), 7.26 (d, *J* = 8.5 Hz, H-5), 7.12 (dd, *J* = 3.0, 9.0 Hz, H-6), 6.90 (d, *J* = 3.0 Hz, H-8), 6.42 (d, *J* = 9.6 Hz, H-3), 3.84 (s, $-OCH_3$). Lit. (Zubia *et al.*, 1992) ^1H NMR data ($CDCl_3$) ppm 7.63 (d, *J* = 9.4 Hz, H-4), 7.37 (d, *J* = 8.5 Hz, H-5), 6.84 (dd, *J* = 2.6, 8.5 Hz, H-6), 6.80 (d, *J* = 2.6 Hz, H-8), 6.24 (d, *J* = 9.4 Hz, H-3), 3.87 (s, $-OCH_3$). ^{13}C NMR data (75 MHz, $CDCl_3$) 161.0 (s), 156.1 (s), 148.5 (s), 143.1 (d), 119.4 (d), 119.2 (s), 117.9 (d), 117.1 (d), 110.0 (d), 55.8 (q); Lit. (Osborne, 1989) ^{13}C NMR data (15 MHz, $CDCl_3$) 163.2 (s), 161.4 (s), 156.2 (s), 143.8 (d), 112.7 (d), 112.7 (s), 129.1 (d), 113.2 (d), 101.1 (d), 55.9 (q); EIMS *m/z* 176 [M^+] (100), 161 (35), 148 (30), 133 (38), 118 (3), 105 (17), 89 (5), 77 (18), 63 (7), 51 (24); Lit. (Zubia *et al.*, 1992) EIMS *m/z* 176 [M^+] (87), 148 (73), 133 (100), 105 (14), 91 (6), 77 (30); HREIMS *m/z* 176.0471 (calcd. for $C_{10}H_8O_3$ 176.0471).



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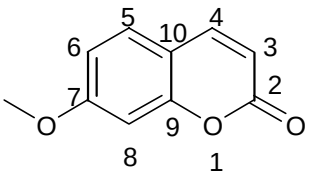

23

4.6 Epicoccamide, a novel secondary metabolite from a jellyfish-derived culture of *Epicoccum purpurascens*

Abstract

From the inner tissue of the jellyfish *Aurelia aurita* a marine sample of the fungus *Epicoccum purpurascens* was obtained. After mass cultivation the fungus was investigated for its secondary metabolite content, and found to contain the new tetramic acid derivative epicoccamide (**24**). The structure of the new compound was elucidated using spectroscopic methods, mainly 1D and 2D NMR and ESI-MS.

Introduction

Fungi investigated from the marine environment are often derived from marine sediments (Toske *et al.*, 1998; Onuki *et al.*, 1998; Cui *et al.*, 1996), wood (Renner *et al.* 1998), or algae (Amagata *et al.*, 1998d; Iwamoto *et al.*, 1998; Belofsky *et al.*, 1998). They are also obtained from marine animals which is reflected by the numerous studies undertaken on sponge-derived fungi, e.g., gymnostatins A-E from *Gymnascella dankaliensis* isolated from the sponge *Halichondria japonica*, A-C of which exhibit significant cytotoxicity against cultured P-388 cells (Amagata *et al.* 1998a). Additionally, the antimicrobial metabolite microsphaeropsisin was isolated from a *Microsphaeropsis* sp., which was associated with the sponge *Myxilla incrustans* (Höller *et al.*, 1999). Further investigations into the secondary metabolite content of fungi derived from marine animals other than sponges have led to reports on *Emericella unguis* isolated from a mollusc and a medusa and found to produce the antibacterial depside guisinol (Nielsen *et al.*, 1999), an unidentified fungus isolated from a soft orange coral which was found to produce the cytotoxic and antimicrobial spiroxins (McDonald *et al.*, 1999), *Pithomyces* sp., derived from the tunicate *Oxycorynia fascicularis* which produced the polyketides pitholides A-D (Wang *et al.*, 1997), *Periconia byssoides*, the source of pericosines A and B which have antitumour activity, from the sea hare *Aplysia kurodai* (Numata *et al.*, 1997a), *Aspergillus fumigatus*, from a marine fish *Pseudolabrus japonicus*, which produced fumiquinazolines A-G (Takahashi *et al.*, 1995b), and *Penicillium fellutanum* which produced the cytotoxic peptides fellutamides A and B (Shigemori *et al.*, 1991) from the gastrointestine of the marine fish *Apogon endekataenia*.

During our studies concerning the isolation of marine-derived fungi and the investigation of their secondary metabolite content, a marine isolate of *Epicoccum purpurascens* could be isolated from the inner tissue of the jellyfish *Aurelia aurita*. Though no obligate marine species of the fungus *Epicoccum* has been described representatives of this genus are found to occur on washed-up algae and other beach wrack material (Kohlmeyer & Volkmann-Kohlmeyer, 1991). Terrestrial species of the genus *Epicoccum* have been studied for their natural product content, e.g., epicorazines A and B (Baute *et al.*, 1978), epirodin (Ikawa *et al.*, 1978), triornicin (Frederick *et al.*, 1981), all being produced by *E. nigrum* (= *E. purpurascens*)

(Domsch *et al*., 1980), indicating some members of the genus *Epicoccum* to have a highly developed and diverse secondary metabolism.

Materials and methods

Isolation and Taxonomy of the fungus

The jellyfish *Aurelia aurita* was collected from the North Sea, Tönning, Germany. Its inner tissue was cut into small pieces and placed on agar plates containing isolation medium (15 g/L agar and 1000 mL sea water from the sample collecting site. After autoclaving the antibiotics benzylpenicillin and streptomycin sulphate were added by sterile filtration). Fungal colonies growing out of the jellyfish tissue were transferred to medium for sporulation (1.0 g glucose, 0.1 g yeast extract, 0.5 g peptone from meat, enzymatic digest, 15 g agar, and 1000 mL sea water, pH 8) in order to enable taxonomy of the isolates.

Cultivation

The microorganism was cultured at 20 °C for 50 days in 4.5 litres of solid malt extract Soya meal agar (30 g malt extract, Merck, 3 g peptone from Soya meal, papain-digest, Merck, 7.6 g agar, 800 mL artificial sea water and 200 mL demineralised water, adjusted to pH 5.5).

Biological activity, see chapter 3.5.

Conditions/Instruments for the MS analysis

Electrospray mass spectrometry (ESI-MS) was performed on a Finnigan TSQ 700 ESI spectrometer. The range of masses investigated was *m/z* 300 > 1000; for MS/MS experiments *m/z* 20 > 600. Collision energies used for the MSMS (+) experiment were -39 eV and -59 eV, for the MSMS (-) experiment it was -44 eV.

Extraction and Isolation

Prior to extraction with 13.5 L EtOAc the solid medium and fungal mycelium were diluted with H_2O and blended using the Ultra Turrax T 25 at 8000 min^{-1}. The resultant EtOAc extract (3.9 g) was purified employing a combination of chromatographic techniques. First, it was passed over normal phase silica (vacuum liquid chromatography, VLC) using a gradient of CH_2Cl_2/EtOAc/MeOH as eluent to yield 13 fractions each of 250 mL. VLC fraction 11 (2.2 g, eluted with MeOH/EtOAc 60:40) was subjected to RP-18 HPLC (Eurospher 100, 5 μm, 8 mm × 25 cm) using MeOH:H_2O, 85:15 as eluent to yield compound **24**.

Results and Discussion

In the present study the isolation and structure elucidation of epicoccamide obtained from *Epicoccum purpurascens* is described. The fungus was cultivated on a solid malt extract Soya meal medium with added artificial sea salt. Successive fractionation of the EtOAc extract by vacuum-liquid chromatography (VLC), and normal (Si-60) and reversed (RP-18) phase HPLC yielded compound **24**.

The molecular formula of epicoccamide (**24**) was deduced to be $C_{29}H_{51}NO_9$ by a combination of electrospray ionisation (ESI) mass spectrometry and NMR spectroscopy. Of the five elements of unsaturation implied by the molecular formula of **24**, two were present as carbonyl groups [δ 197.6 (C-3, s), 201.3, (C-7, s)], and one as an α, β unsaturated alcohol [δ 175.4 (C-1, s)], as deduced from the ^{13}C NMR and IR spectra of the compound. This deduction enabled three of the nine oxygen atoms to be accounted for and also indicated the molecule to be bicyclic since no other multiple bonds were present. From the ^{1}H and ^{13}C NMR data of **24** (Table 17) it was evident that the molecule was composed of three distinct fragments; a mannose, an aliphatic chain, and a tetramic acid moiety. After all protons had been associated with their directly bonded carbon atoms *via* a ^{1}H-^{13}C 2D NMR shift-correlated measurement (HMQC) it was possible to develop the mannose and aliphatic moieties from their ^{1}H-^{1}H COSY data. Thus, cross-peaks between the resonance for H-1', the mannose anomeric proton, and H-2', between the resonances for H-2' and H-3', those for H-3' and H-4', H-4' and H-5', and between

the resonance for H-5' to those for H_2-6', completed the ^{1}H-^{1}H spin system within the mannose moiety and showed C-1' to be bonded to C-2', C-2' to C-3', C-3' to C-4', C-4' to C-5', and C-5' to C-6'. From the magnitude of the C-H coupling constant between C-1' and H-1' (J = 156.4 Hz) it was evident that **24** contained a β-mannose moiety. Usually, pyranoses with a hydroxyl group at C-1' give J_{CH} values around 160 Hz for the β-form and 170 Hz for the α-form (Bock *et al.*, 1973). The absolute configuration of mannose (D or L) was not determined. It was also evident from the ^{13}C NMR chemical shift of C-1' (101.7 ppm) that the glycosidic linkage to the rest of the molecule was bonded over the oxygen to this atom. The mannose moiety also accounted for all of the remaining oxygen within the molecule as well as for one of the two rings. Remaining to be accounted for were three methyl, 14 methylene, and three methine groups as well as a nitrogen as deduced from the MS, and ^{1}H and ^{13}C NMR data of **24**. Evident in the ^{13}C NMR spectrum of epicoccamide were four broad resonances [δ 201.3 (C-7, s), 197.6 (C-3, s), 175.4 (C-1, s), 101.6 (C-2)] which were best assigned to a tetramic acid moiety (Matsunaga *et al.*, 1991; Sakuda *et al.*, 1996). From the ^{1}H-^{1}H COSY cross-peaks observed between the resonances for H_3-5 and H-4, and between the resonances for H_3-23 and H-8, and between the resonances for H-8 and H_2-9; it was evident that CH_3-5 bonded to C-4, and CH_3-23 bonded to C-8 which is further bonded to C-9. As the resonance for C-8 is also broad in the ^{13}C NMR spectrum it is likely that this group is affected by the keto-enol tautomerism of the tetramic acid moiety (Fig. 10).

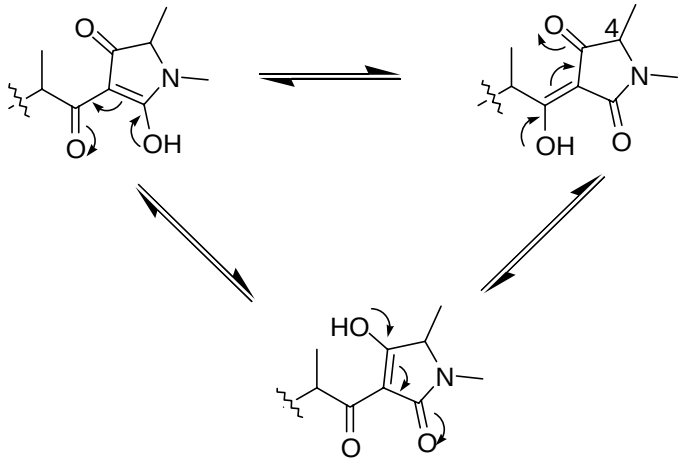

Figure 10. Tautomerism of the tetramic acid moiety.

Table 17. ^{1}H (CD_3OD, 600 MHz) and ^{13}C NMR (CD_3OD, 150 MHz) data[a] for epicoccamide (**24**).

position	δ C	type[b]	δ H	J_{CH}
1	175.4	s	-	
2	101.6	s	-	
3	197.6	s	-	
4	61.8	d	3.54 (br)	
5	16.0	q	1.31 (d, *J* = 6.9 Hz)	
6	26.7	q	2.91 (brs)	
7	201.3	s	-	
8	40.8	d	3.80 (m)	
9	35.0	t	1.27 (m)	
			1.72 (m)	
10-19	30.6-30.9	t	1.25-1.38	
20	27.3	t	1.42 (m)	
21	30.8	t	1.65 (m)	
22	70.6	t	3.57 (ddd, *J* = 2.9/6.8/9.8 Hz)	
			3.98 (m)	
23	18.0	q	1.03 (d, *J* = 6.2 Hz)	
1’	101.7	d	4.54 (brs)	J_{CH} = 156.4 Hz
2’	72.5	d	3.90 (brd, *J* = 3.0 Hz)	J_{CH} = 145.9 Hz
3’	75.3	d	3.50 (dd, *J* = 3.0/9.5 Hz)	J_{CH} = 142.0 Hz
4’	68.5	d	3.62 (dd, *J* = 9.5/9.5 Hz)	J_{CH} = 146.2 Hz
5’	78.1	d	3.26 (ddd, *J* = 2.8/5.6/9.5 Hz)	J_{CH} = 140.5 Hz
6’	62.8	t	3.77 (dd, *J* = 5.6/12.6 Hz)	J_{CH} = 143.5 Hz
			3.95 (dd, *J* = 2.8/12.6 Hz)	

[a] All assignments are based on extensive 1D and 2D NMR experiments.

[b] Attached protons as determined by DEPT (C = s, CH = d, CH_2 = t, CH_3 = q).

In the ^{1}H NMR spectrum of **24** the signal for H-8 (δ 3.80, m) indicated it to be deshielded relative to protons adjacent to "normal" carbonyl groups, a characteristic which is typical of such systems (Kanazawa *et al.*, 1993). H_3-6 was assigned to a N-CH_3 on the basis of its ^{1}H and ^{13}C NMR chemical shifts. Finally, long-range ^{1}H-^{13}C 2D NMR correlations (HMBCs) (Figure 13) observed between the resonances for H_3-6 and C-1 and C-4; between those associated with H_3-5 and C-3 and C-4, and between those of H_3-23 and C-2, C-7, C-8 and C-9, clearly showed C-4 and C-1 to bond to the N-CH_3, C-3 to bond to C-4, C-7 to bond to C-8 and C-2, and by deduction C-2 to bond to both C-1 and C-3, thus completing the tetramic acid moiety and the second ring within the molecule. This left 14 methylene groups to be assigned; these were deduced to be present as a single aliphatic chain connected to C-8 at one end and to the mannose *via* C-22 at the other. From the long-range correlation observed between the resonances for H_2-22 and that of C-1' it was obvious that the aliphatic chain was linked to the anomeric carbon C-1'. The electrospray (ESI) mass spectrum (negative mode) of epicoccamide contained the highest mass signal at *m/z* 556 consistent with $[M-H]^-$. In contrast, collision-induced dissociation-tandem mass spectrometry (CID-MS/MS, negative mode) showed the 556 peak and a base peak at *m/z* 394 $[M\text{-mannose}]^-$ (see Fig. 11). Additionally, a *m/z* 126 peak present in the ESI CID-MS/MS (negative mode) results from the keto-form of the tetramic acid moiety (see Fig. 11) cleaving from the molecule. In the ESI (+) mass spectrum *m/z* 580 ($[M+Na]^+$) is the base peak. A CID-MS/MS (+) shows the formation of a *m/z* 418 ion as base peak which represents the sodium adduct of **24** less the mannose. By increasing collision energy from -39 eV to -59 eV a loss of water from the molecule was observed (base peak *m/z* 400). All of the MS data are in good agreement with the proposed structure of **24**, epicoccamide.

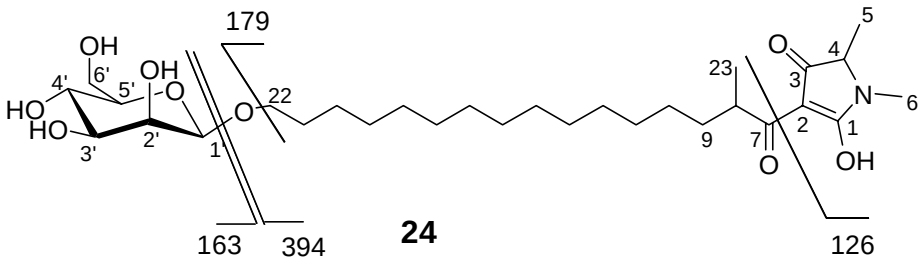

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Figure 11. ESI-CID-MS/MS fragments of **24**.

A number of natural products with a tetramic acid moiety are known, e.g., aflastatin A (**I**) produced by a *Streptomyces* sp. (Table 18) was found to inhibit aflatoxin biosynthesis (Sakuda *et al.*, 1996). The tetramic acid glycosides aurantosides A (**II**) and B from the marine sponge *Theonella* sp., show cytotoxicity towards P-388 and L-1210 leukaemia cells (Matsunaga *et al.*, 1991). From the sponge *Halichondria cylindrata* cylindramide (**III**), a tetramic acid lactam, was isolated which had cytotoxic effects towards B 16 melanoma cells (Kanazawa *et al.*, 1993). Fischerellin A (**IV**), isolated from a culture of the cyanobacterium *Fischerella muscicola* (Hagmann & Jüttner, 1996) had antifungal, and herbicidal activities as well as being a potent photosystem-II-inhibitor. As many of the reported tetramic acid derivatives exhibited pharmacological activities, in particular cytotoxic activity, it was surprising to find that epicoccamide had no detectable activity in any of the applied assays.

Epicoccamide (**24**) was obtained as white amorphous powder (2.6 mg/L); $[\alpha]^{20}_{D}$ = -10.3° (*c* = 0.10, EtOH); UV (EtOH) λ_{max} (log ε) 279 (4.90) nm; IR (film) ν_{max} 3360, 2925, 2850, 1655, 1595, 1480 cm^{-1}; ^{1}H and ^{13}C NMR data see Table 17; ESI-MS (-): *m/z* 556 $[M-H]^-$; (+): 580 $[M+Na]^+$; CID-ESI-MS/MS (-): 556 (31), 394 (100), 126 (40); (+): 418 (28), 400 (100).

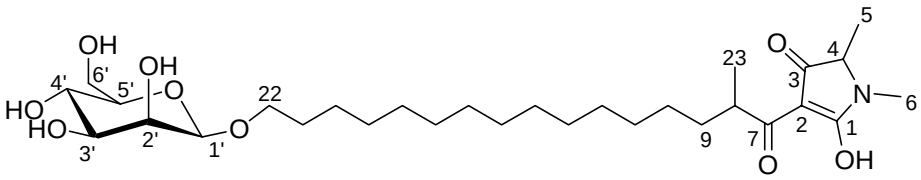

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Table 18. ^{13}C data of different tetramic acid moiety containing natural products recently published being compared to epicoccamide (**24**).

position	δ C of epicoccamide[a]	δ C of **I**[b]	δ C of **II**[c]	δ C of **III**[d]	δ C of **IV**[e]
1	175.3	175.8	174.8	176.7	172.8
2	101.6	101.0	102.0	101.2	91.6
3	197.6	196.0	195.0	194.3	198.8
4	61.8	61.8	65.5	68.8	61.8
5	16.0	16.3	-	-	15.3
6	26.7	26.9	-	-	26.3
7	201.3	195.5	176.1	190.5	173.7

[a] CD_3OD, 150 MHz.

[b] CD_3OD, 125 MHz.

[c] CD_3OD.

[d] CD_3OD,/$CDCl_3$ 1:1, 150 MHz.

[e] $CDCl_3$.

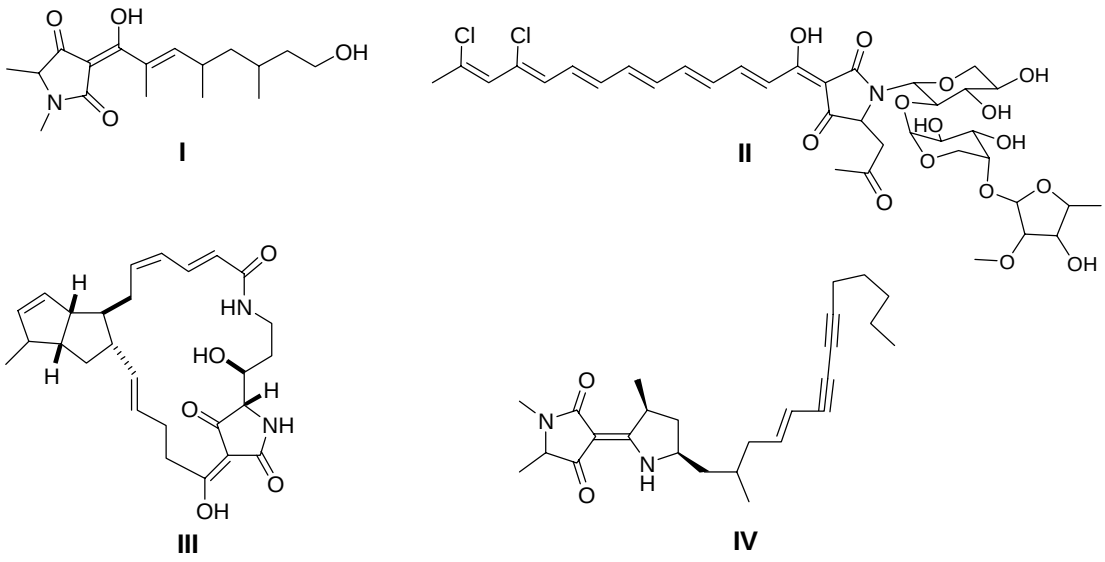

Figure 12. Tetramic acid moieties containing derivatives **I**-**IV**.

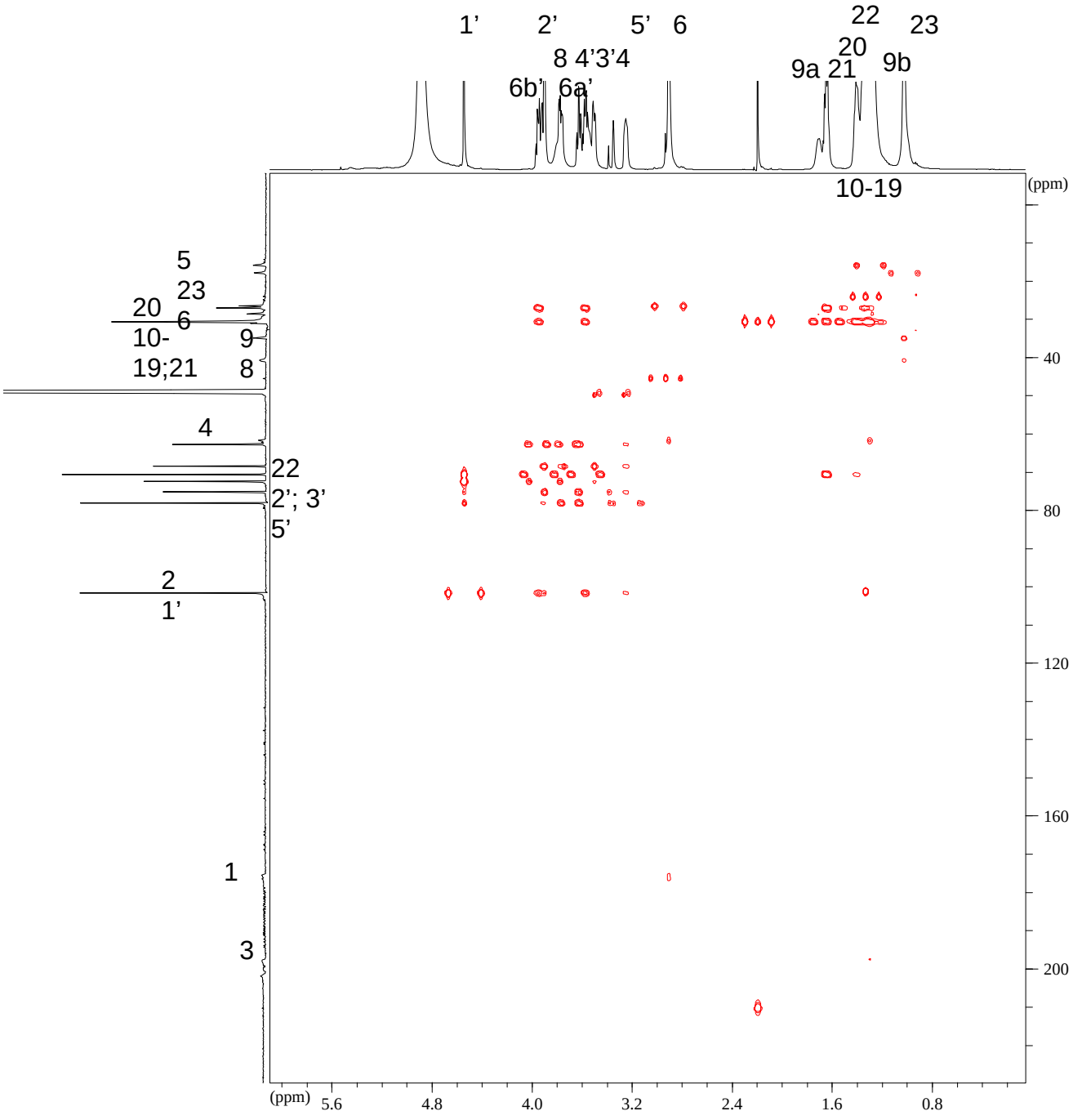

Figure 13. ^{1}H-^{13}C long-range (HMBC) NMR spectrum of **24**; CD_3OD, 600 MHz for ^{1}H, 150 MHz for ^{13}C.

5 Summary

From 56 algal samples, 6 marine plants or plants from the intertidal zone, a sponge and a jellyfish a total of 428 fungal strains was isolated, representing 45 genera (6 genera of Ascomycetes, 2 genera of Zygomycetes, 37 genera of Mitosporic fungi), and 110 strains of Mycelia sterilia. The three obligate marine fungi isolated were *Ascochyta salicorniae, Corollospora maritima* and *Dendryphiella salina*.

In a comparative study, ethyl acetate extracts of terrestrial and marine *Phoma* spp., were analysed using RP-HPLC coupled with diode-array detection (DAD) and thermospray mass spectrometry (TSP-MS). Statistical interpretation of retention times, UV- and mass spectral data allowed 84 % of all of the extracts to be confirmed as being derived from either the terrestrial or marine samples. This finding suggests that marine-derived *Phoma* spp., differ significantly from terrestrial *Phoma* spp., with respect to their secondary metabolite content.

In order to investigate their biologically active secondary metabolite production, 265 fungal strains were chosen for small scale cultivation on three different media. EtOAc extracts of 222 strains (83.8 %) showed antimicrobial activity against at least one of used test organisms in agar diffusion assays for antibacterial, antifungal and antialgal activity. EtOAc extracts of 138 strains (52.1 %) showed activity against *Artemia salina*. EtOAc extracts of 26 fungal strains (9.8 %) were fatal towards *Caenorrhabditis elegans*. EtOAC extracts of 43 strains were further tested: HIV-1 reverse transcriptase was inhibited by extracts of six strains (14.0 %), and tyrosine kinase by extracts of 37 strains (86.0 %). Of 9 extracts tested one extract (11.1 %) showed significant cytotoxicity towards L-6 cells, two extracts (22.2 %) were active against *Plasmodium falciparum*, and two (22.2 %) exhibited inhibition of *Trypanosoma brucei* ssp. *rhodesiense*, three strains (33.3 %) against *Trypanosoma cruzi*, one strain of them a prominent inhibition against both *Trypanosoma* species.

Extracts of 7 fungal strains, including five algal-derived and two jellyfish-derived strains, were investigated chemically. This investigation resulted in the isolation and structure elucidation of 24 pure compounds representing secondary metabolites. 15

compounds proved to be new: the unprecedented and structurally unusual tetramic acid containing metabolite ascosalipyrrolone A (**1**) has antiplasmodial activity towards *Plasmodium falciparum* strains K1 and NF 54. It also shows antimicrobial activity and inhibits tyrosine kinase $p56^{lck}$. Additionally, ascosalipyrone (**3**) was isolated from the obligate marine fungus *Ascochyta salicorniae*; the sesquiterpenoid compounds drechslerines A-D (**6**-**8**, **13**), 9-hydroxyhelminthosporol (**10**), drechslerones A (**11**) and B (**12**), drechslepoxides A (**14**) and B (**17**) and drechsleral (**15**) from the algicolous fungus *Drechslera dematioidea*. Drechslerine C (**8**) and drechsleral (**15**) have prominent antiplasmodial activity towards *Plasmodium falciparum* strains K1 and NF 54. The algicolous fungus *Phoma tropica* contained the new natural product 5-hydroxyramulosin (**22**), and the jellyfish-derived culture of *Epicoccum purpurascens* was found to produce the unusual tetramic acid moiety containing compound epicoccamide (**24**). The known compounds genistein (**4**), 2,3-dihydro-2-hydroxy-2,4-dimethyl-5-trans-propenylfuran-3-one (**5**), helminthosporol (**9**), *cis*-sativenediol (**16**), drechslerine E [known as (+)-secolongifolene] (**18**), isocochlioquinones A and C (**19** and **20**), cochlioquinone B (**21**) and 7-methoxycoumarin (**23**) were isolated as well.

6 General discussion and future prospects

The discussion of the partial projects is placed after the respective chapters. Here some general aspects and future prospects shall be mentioned.

As illustrated in Chapter 1.2 marine-derived fungi are a source for novel natural products. However, the hope of finding secondary metabolites with unprecedented structures completely differing from those isolated from terrestrial fungi could not be met. This was clearly demonstrated by, e.g., the study of Höller (dissertation, 1999) who investigated the secondary metabolites of sponge-derived fungi. Of 39 compounds isolated 33 had known structures which were closely related to those of metabolites of terrestrial fungi. Thus, in order to find a higher percentage of compounds with unique structures, it was decided to investigate fungi of another ecological niche.

The current study thus focused on the investigation of endophytic fungi from marine algae. Hardly any literature data on what type of fungi were isolated from algae (Kohlmeyer, 1974; Kohlmeyer & Kohlmeyer, 1979; Hawksworth, 1988; Stanley, 1992) and only very few data on secondary metabolites from algal derived fungi (see Chapter 1.2 and literature cited there) are available. Using a special isolation technique which has been used for the isolation of endophytes from higher plants (Petrini *et al.*, 1992; Schulz *et al.*, 1993), this study showed for the first time which fungal genera are to be found in marine alga (Table1) and what secondary metabolites they produce.

According to Schulz *et al.* (1998) fungal endophytes can colonize internal plant tissues without causing apparent harm to their host. There must be some interaction between host plant and fungal endophyte. The results of Schulz *et al.* (1999a) screening 4000 isolates from plants and soils revealed that only 18 % of the fungal isolates from soils showed herbicidal activity, but 52 % of those from plants did. The endophytes were responsible for the high activity among the plant isolates, with 57 % of the strains inhibiting at least one of the test organisms for antialgal/herbicidal activity, in contrast only 27 % of the phytopathogens and 25 % of the epiphytes

produced herbicidally active secondary metabolites. Petrini (1992) reported that endophytic fungi produce antibiotic compounds in culture which are active against human and plant pathogenic bacteria. Many endophytes protect their host plants from natural enemies which indicates these fungi to have certain chemical strategies against herbivores (Caroll, 1988). In the current project, biological investigations of crude extracts of selected cultures of endophytic fungi showed in analogy to the report of Schulz *et al.* (1999b) that they possess an enormous biosynthetic potential, especially for the production of antimicrobially active metabolites (Chapter 4.1). This is best illustrated by the isolation of compound **1** which proved to be a potently active antimicrobial. Our results show that fungal endophytes represent an interesting research subject and justify further chemical investigations of this source.

The current study revealed that a great taxonomical variety of fungi is encountered in marine algae (chapter 4.1). Kohlmeyer & Kohlmeyer (1979) isolated many fungi associated with algae; most of them were obligate marine species. Surprisingly, only three obligate marine fungi, i.e. *Ascochyta salicorniae, Corollospora maritima* and *Dendryphiella salina* could be obtained in the current study. This difference may be explained by the different isolation methods. Kohlmeyer & Kohlmeyer used direct isolation methods, and Volkmann-Kohlmeyer & Kohlmeyer (1993) also described the direct isolation method as the best approach to isolate obligate marine strains.

Since many fungi belonging to genera well known for terrestrial fungi were obtained during this (chapter 4.1) and other (Höller, dissertation 1999) studies, the question whether marine-derived fungi of genera also known for the terrestrial environment do differ concerning their biosynthetic capabilities needed to be addressed. A study (chapter 4.2) with extracts of terrestrial and marine-derived *Phoma* spp. was performed analysing them for their metabolite content in analogy to Smedsgaard & Frisvad (1996) who investigated mass profiles of *Penicillium* species. Our study allowed 84 % of all of the extracts to be confirmed as being derived from either the terrestrial or marine samples suggesting that marine-derived *Phoma* spp. differ significantly from terrestrial *Phoma* spp. with respect to their metabolism and the resulting natural products. Even though the current study only addressed the genus *Phoma*, it can be assumed that fungi from other genera behave similar.

The investigation of the secondary metabolite content of some marine fungal strains showed the chemical structures of the compounds isolated in the current study to be diverse, i.e. to belong to many different structural classes. From a total of 24 isolated compounds 15 represented new natural products, e.g., the unprecedented tetramic acid derivatives ascosalipyrrolones A (**1**) and B (**2**) (chapter 4.3), epicoccamide (**24**) (chapter 4.6), and the sesquiterpenoid compounds from *Drechslera dematioidea* (chapter 4.4). Several of the natural products exhibited biological activities which merit further pharmacological evaluation. These results back up further investigations on the field of endophytic fungi from marine sources. This is also supported by the report of Dreyfuss & Chapela (1994) describing endophytic, fresh-water and marine fungi as diverse, underexploited fungal groups.

In order to exploit the full biosynthetic potential it might be successful to experiment with culture conditions on a limited number of "creative" strains. The restriction to a few nutrient media as done in the current study is surely a disadvantage. It may thus be a promising approach to select some fungal strains and to optimise their secondary metabolite production by variation of media components, e.g., the use of marine ingredients or by adding toxic compounds to the medium, e.g., antifungals, in low concentrations. Variation concerning the biological activities of tested crude extracts dependent on the medium was often shown (Chapter 8.2), indicating a variation of secondary metabolite production as well.

Additionally, for fungi which secondary metabolites are already known, feeding experiments are possible using precursors as building blocks for new products. Applied to the results of the current study, an interesting approach would be to investigate the ascosalipyrrolone production of the fungus *Ascochyta salicorniae* dependent on medium composition. Marine ingredients such as algal or sponge materials could be supplemented to the cultivation medium, similar to the media used for the isolation of fungi (see 3.4). For feeding experiments, *Ascochyta salicorniae* would be an interesting object as well. By supplementing precursors to the cultivation medium, e.g., different amino acids, probably different ascosalipyrrolone derivatives could be obtained. These could be used for investigations of structure-activity-relationships.

Another possibility, also based on the selection of some promising strains, is to try to influence them by other means than medium composition, e.g., UV-light irradiation, temperature and oxygen variations. This might induce stress to the fungus resulting in the production of new metabolites in order to secure its survival.

7 References

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8 Appendix

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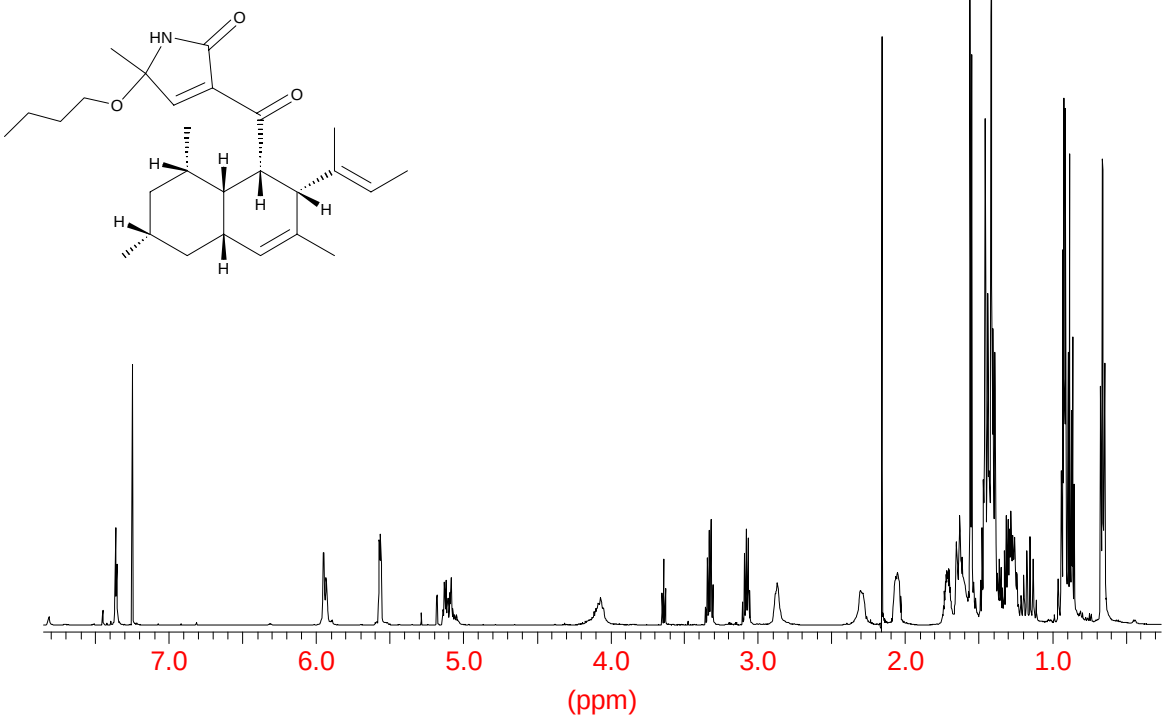
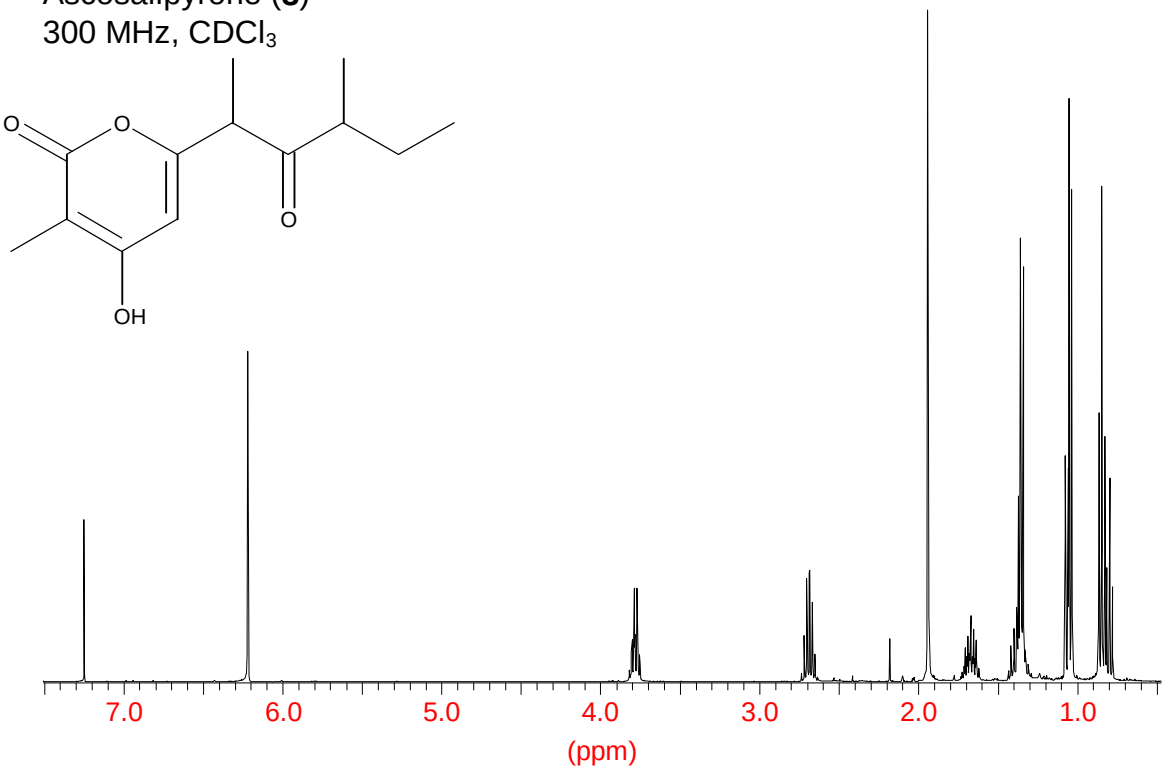
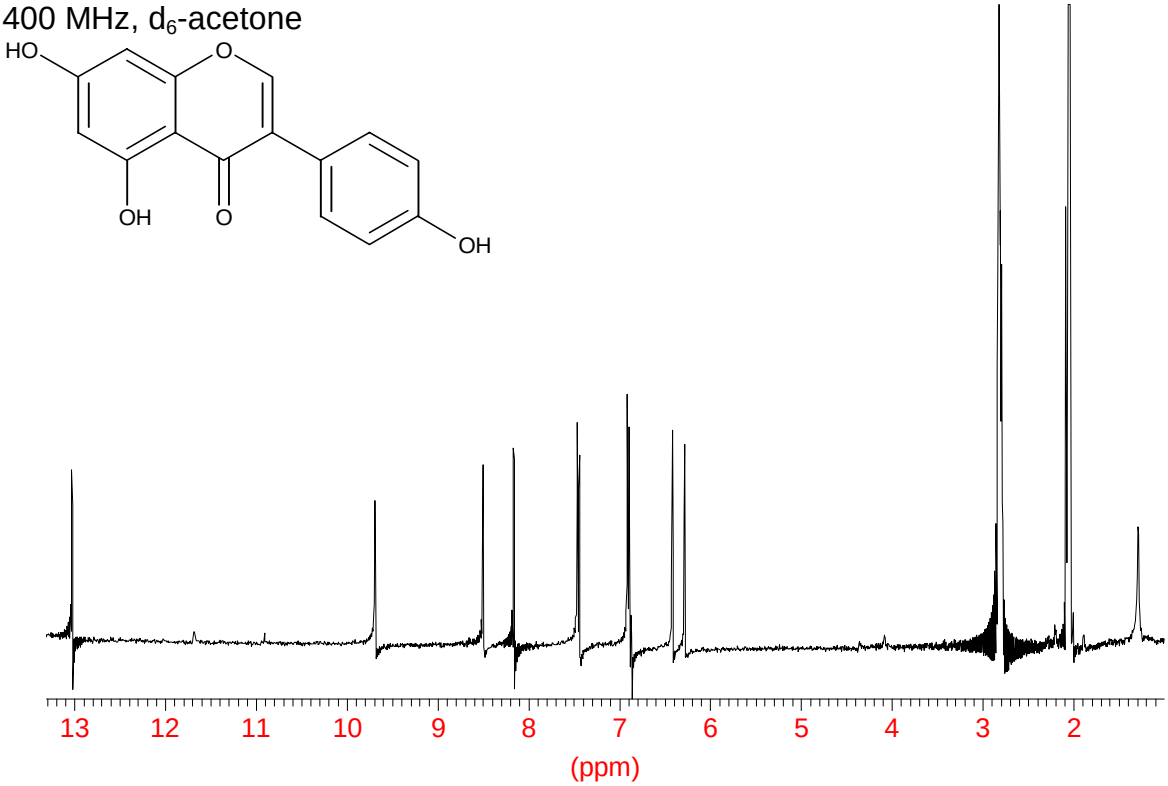
Ascosalipyrrolone A (**1**)
600 MHz, $CDCl_3$



Ascosalipyrone (**3**)
300 MHz, $CDCl_3$



Genistein (**4**)
400 MHz, d_6-acetone



2,3-dihydro-2-hydroxy-2,4-dimethyl-5-trans-propenylfuran-3-one (**5**)
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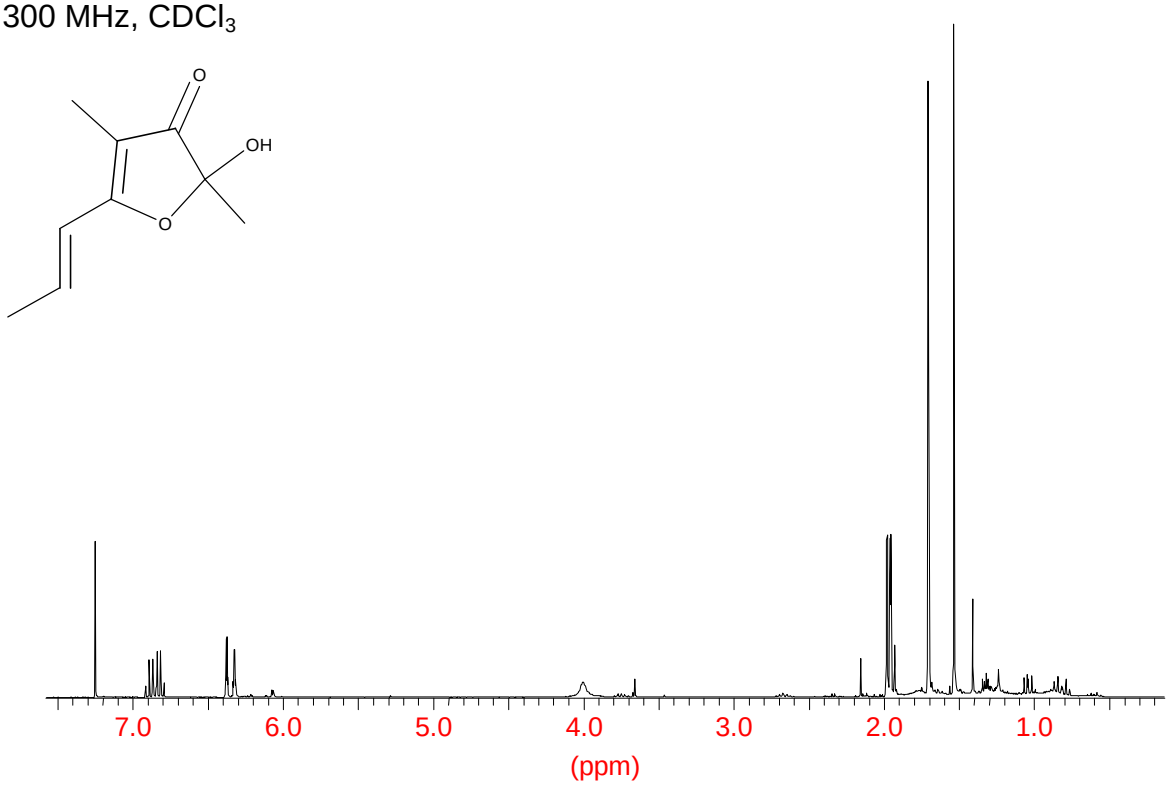
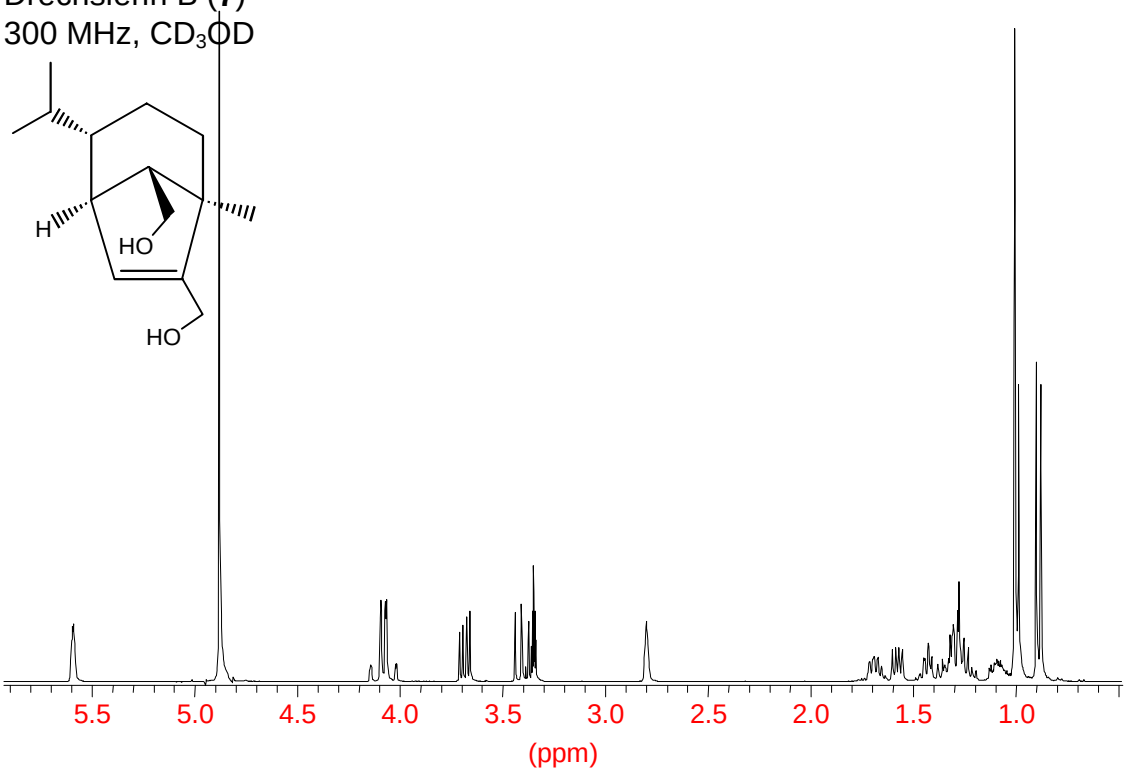
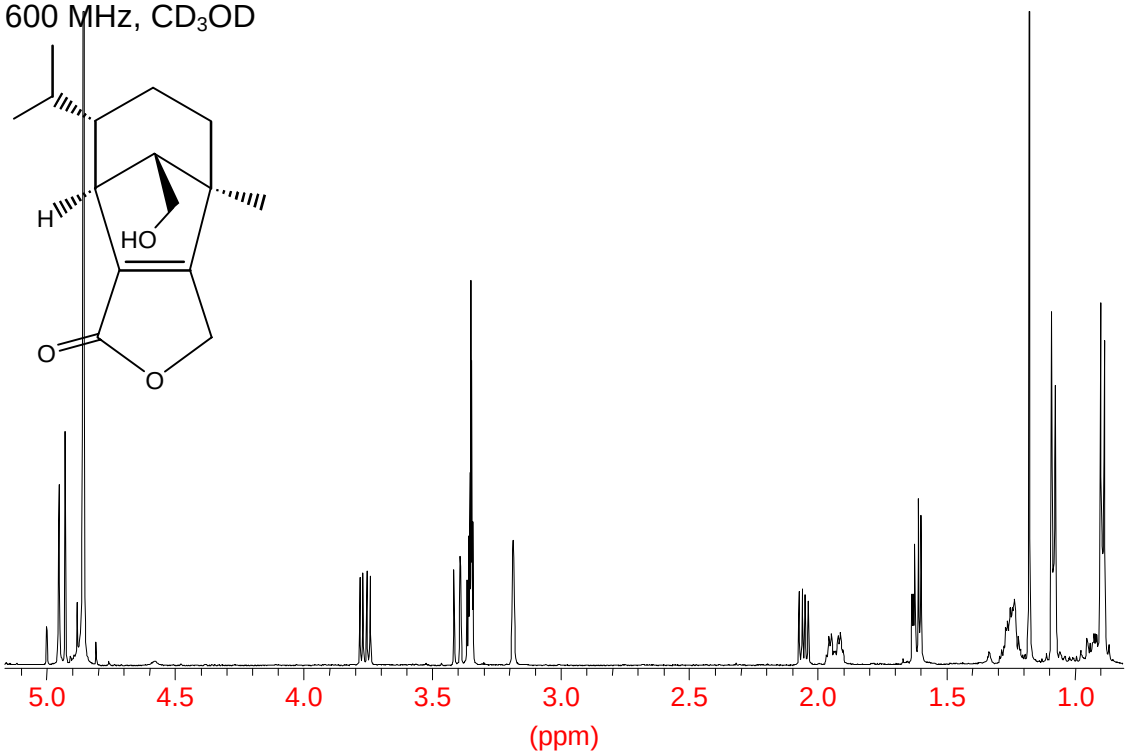


Drechslerin A (**6**)
400 MHz, CD_3OD

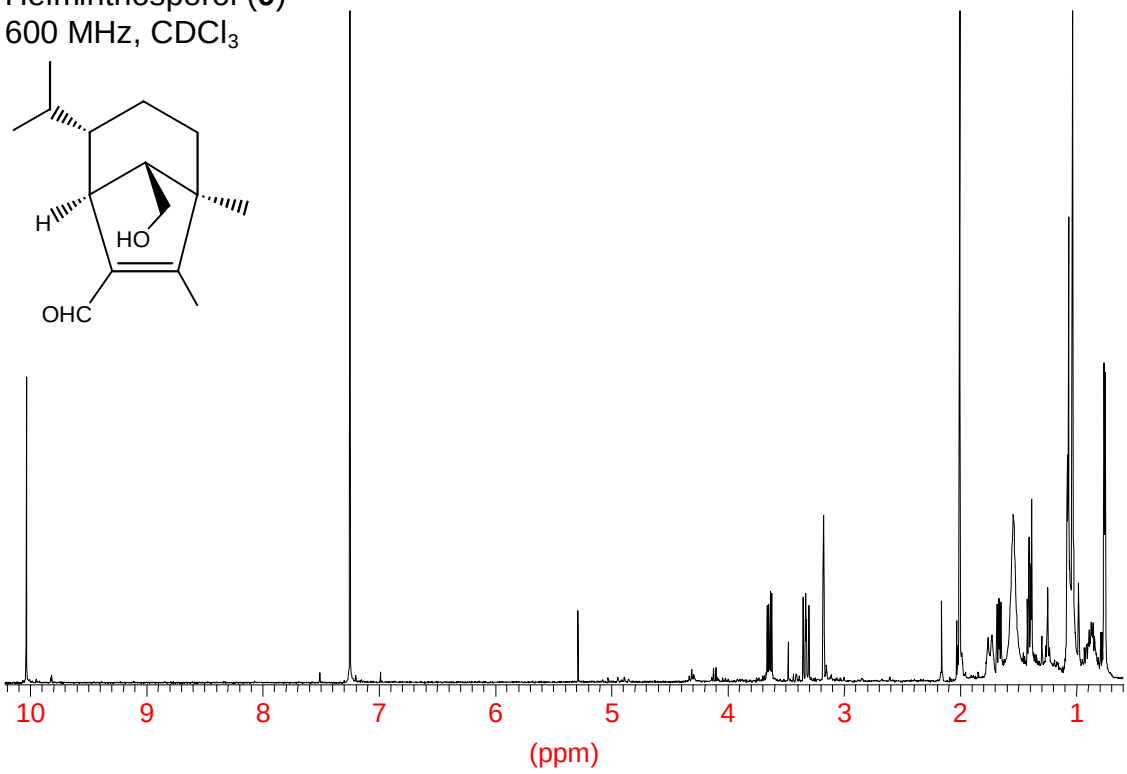
Drechslerin B (**7**)
300 MHz, CD_3OD



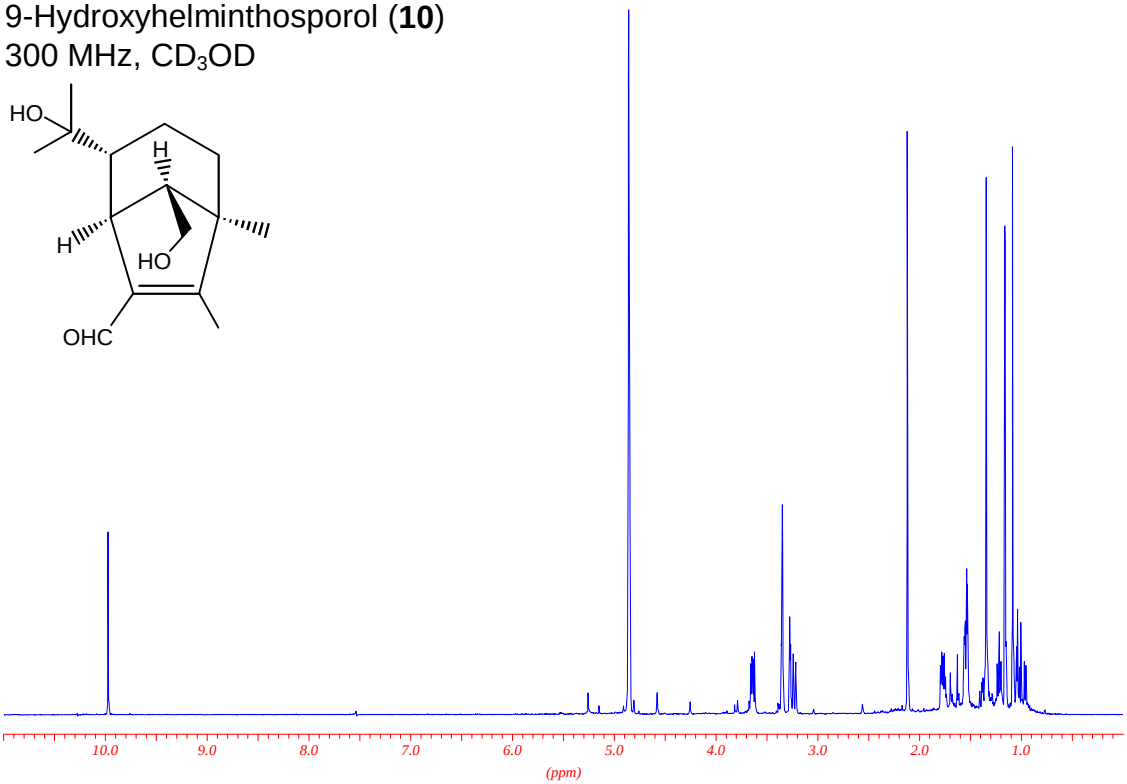
Drechlerin C (**8**)
600 MHz, CD_3OD



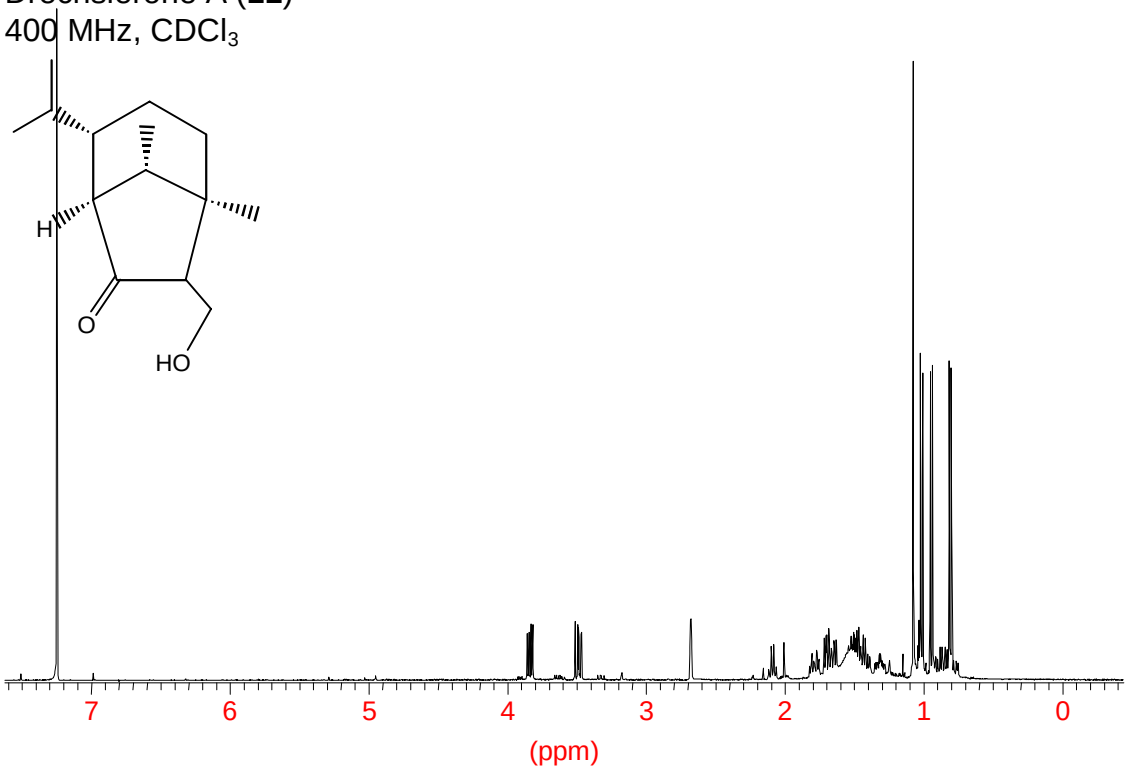
Helminthosporol (**9**)
600 MHz, $CDCl_3$



9-Hydroxyhelminthosporol (**10**)
300 MHz, CD_3OD



Drechslerone A (**11**)
400 MHz, $CDCl_3$



Drechslerone B (**12**)
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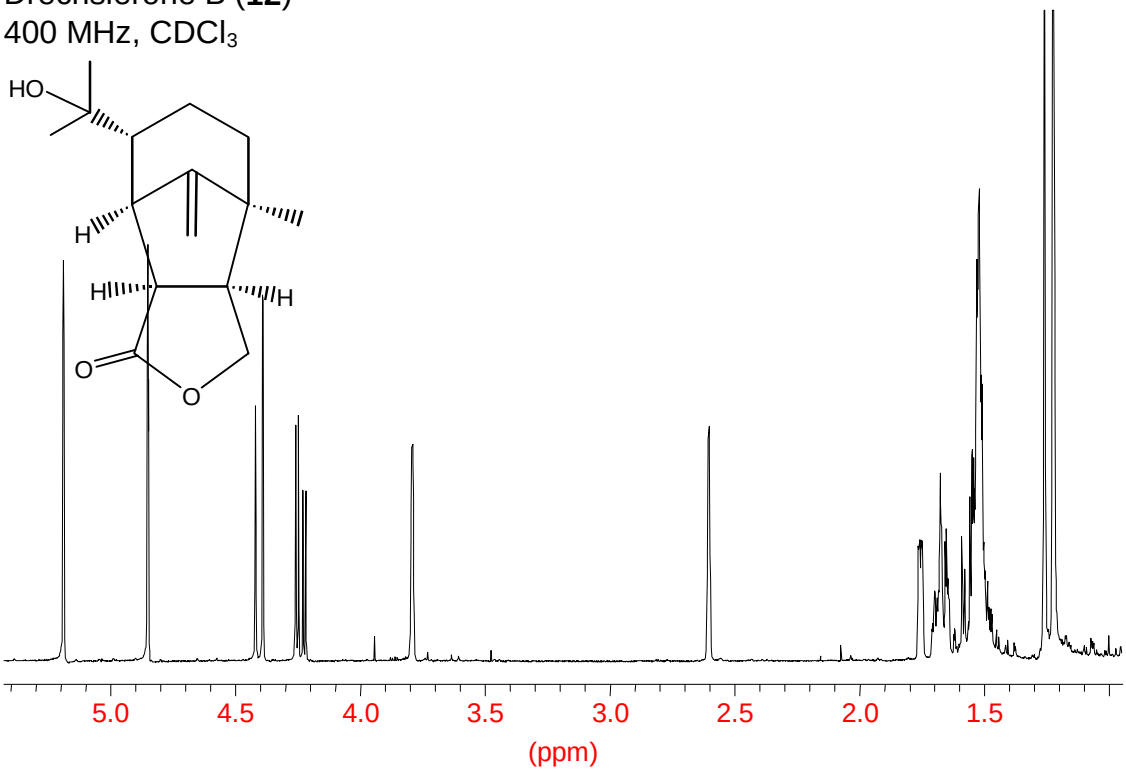

Drechslerin D (**13**)
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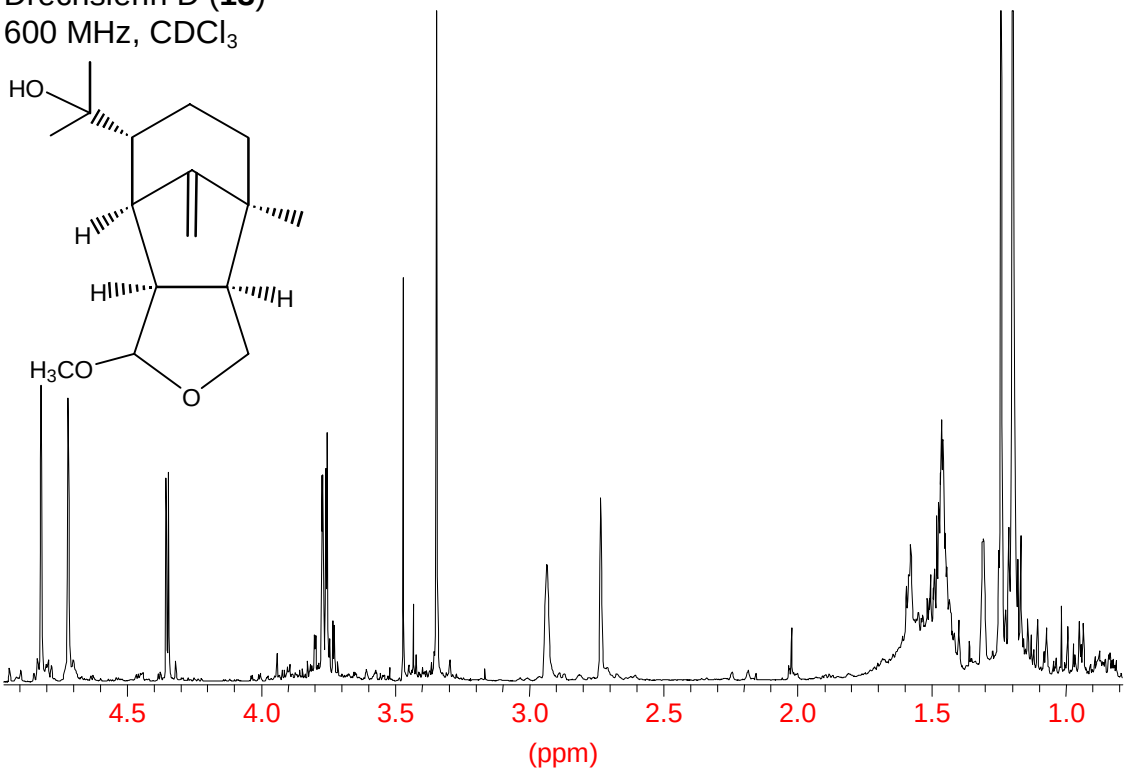
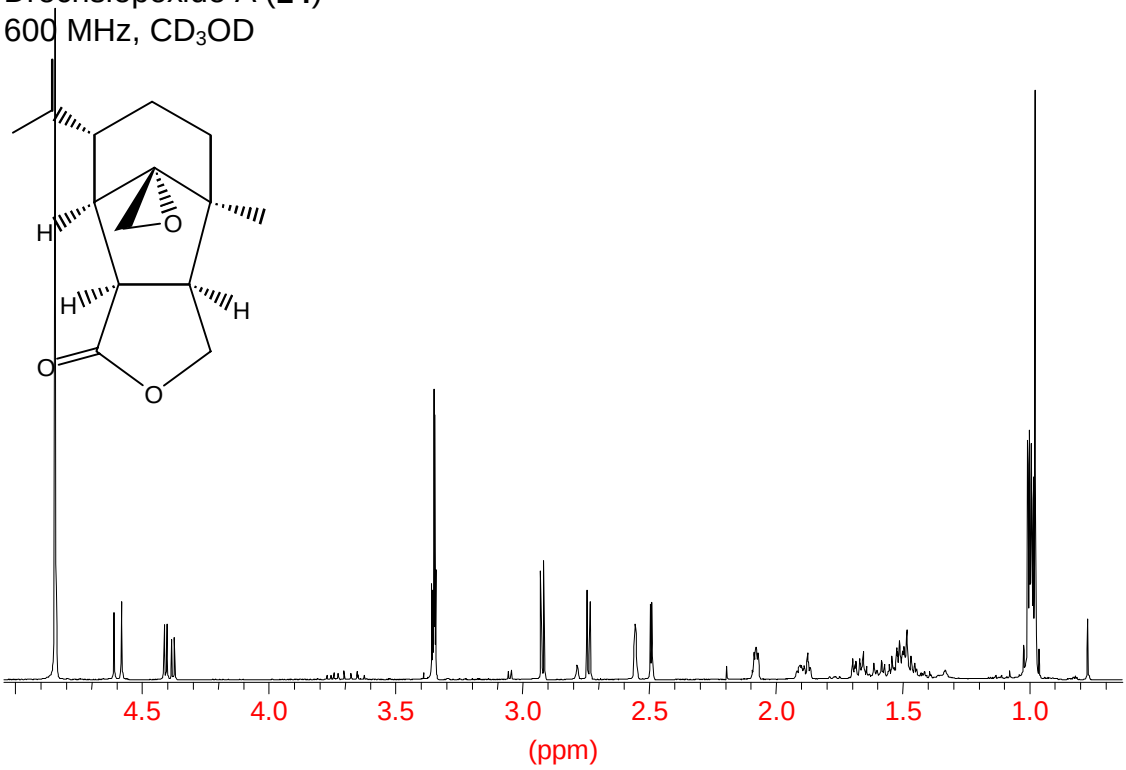


Drechslepoxide A (**14**)
600 MHz, CD_3OD



Drechsleral (**15**)
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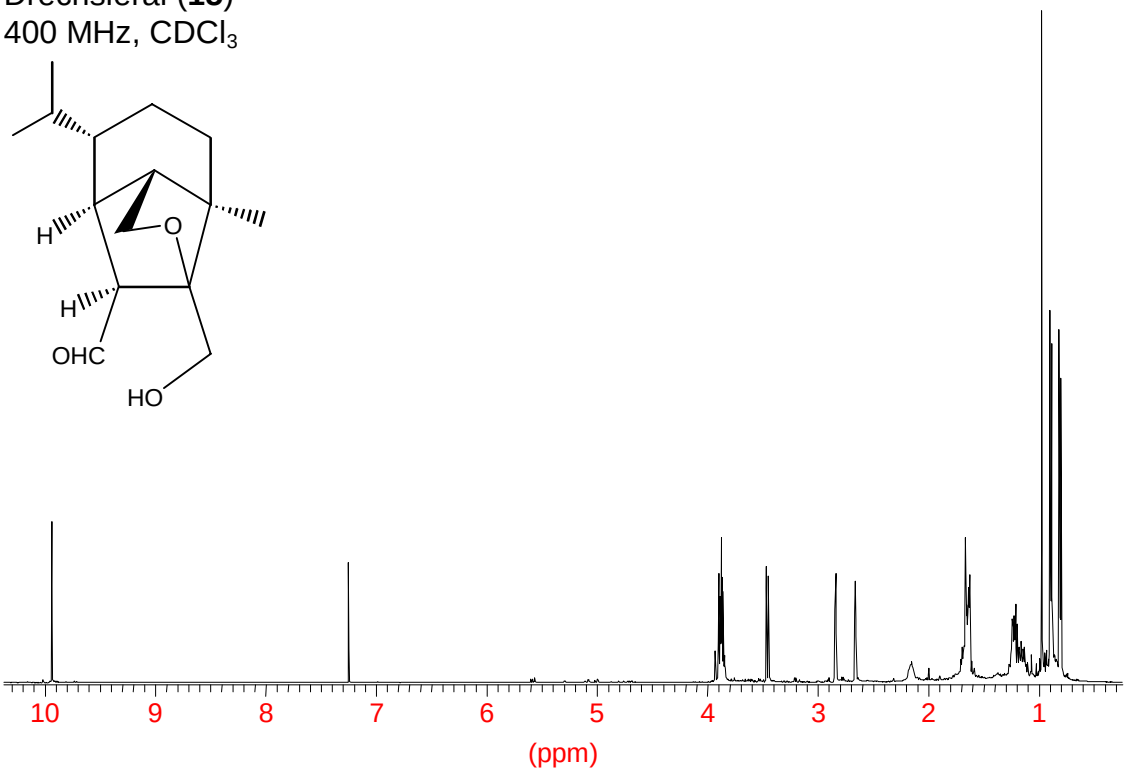

cis-Sativenediol (**16**)
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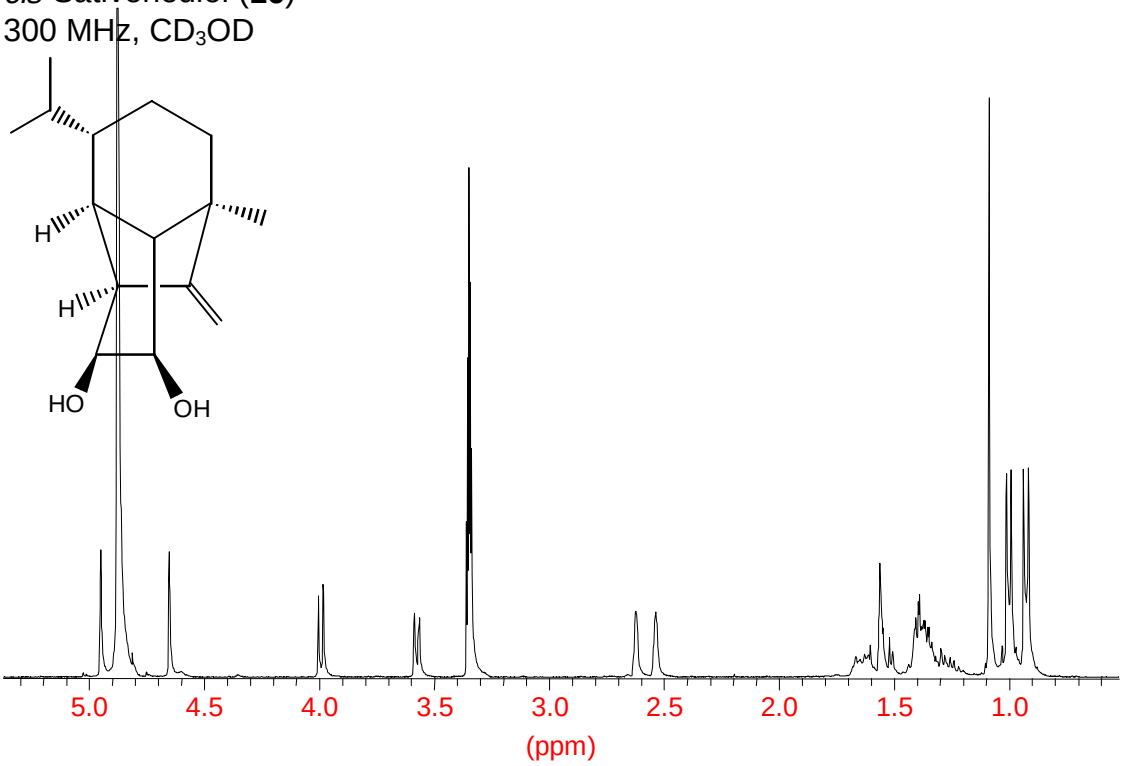

Drechslepoxide B (**17**)
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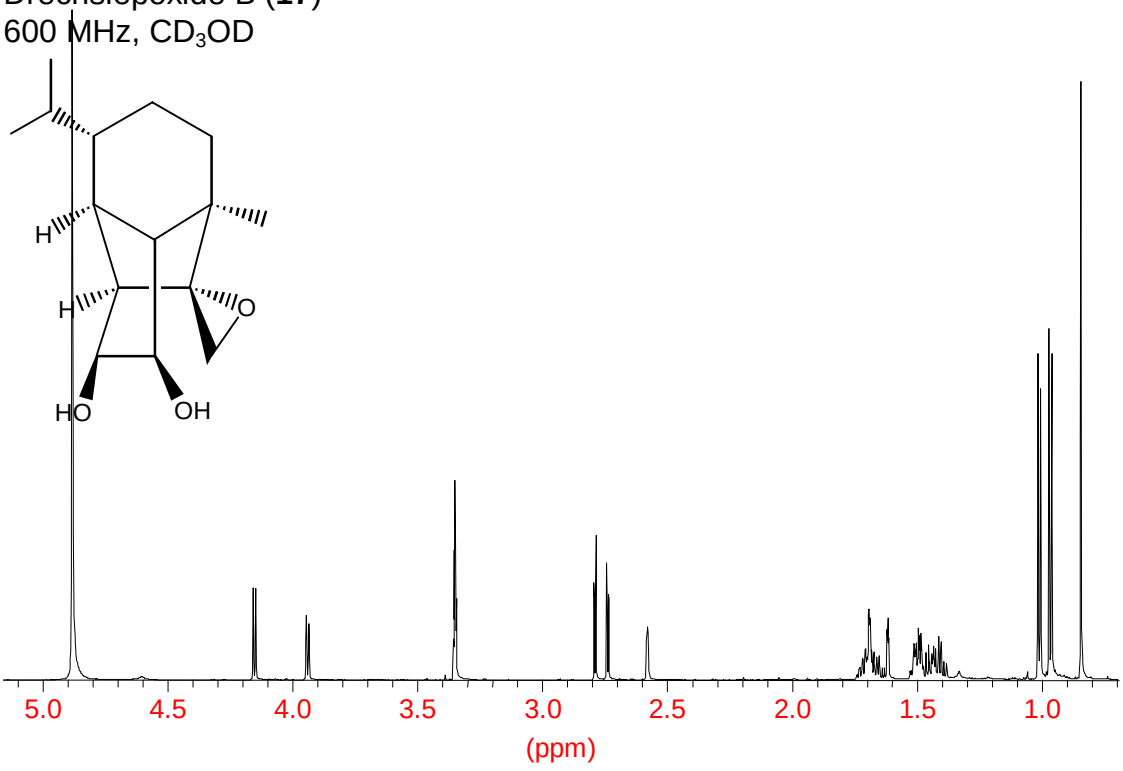

Drechslerin E (**18**)
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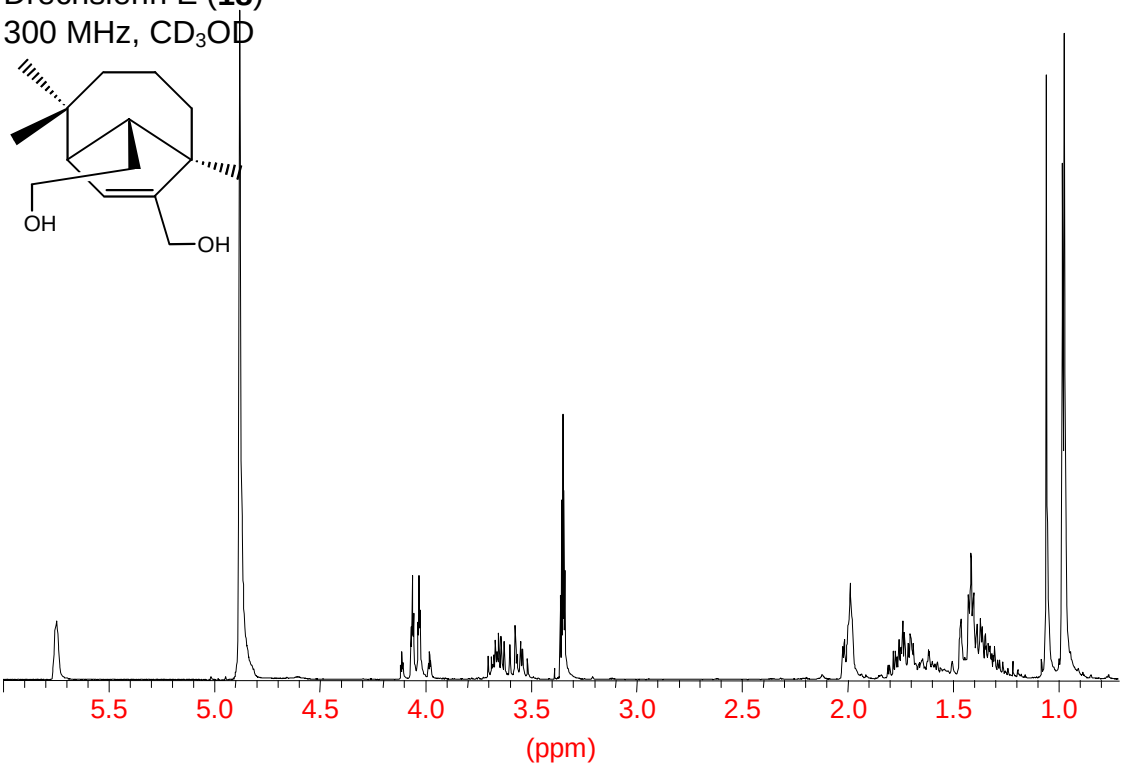
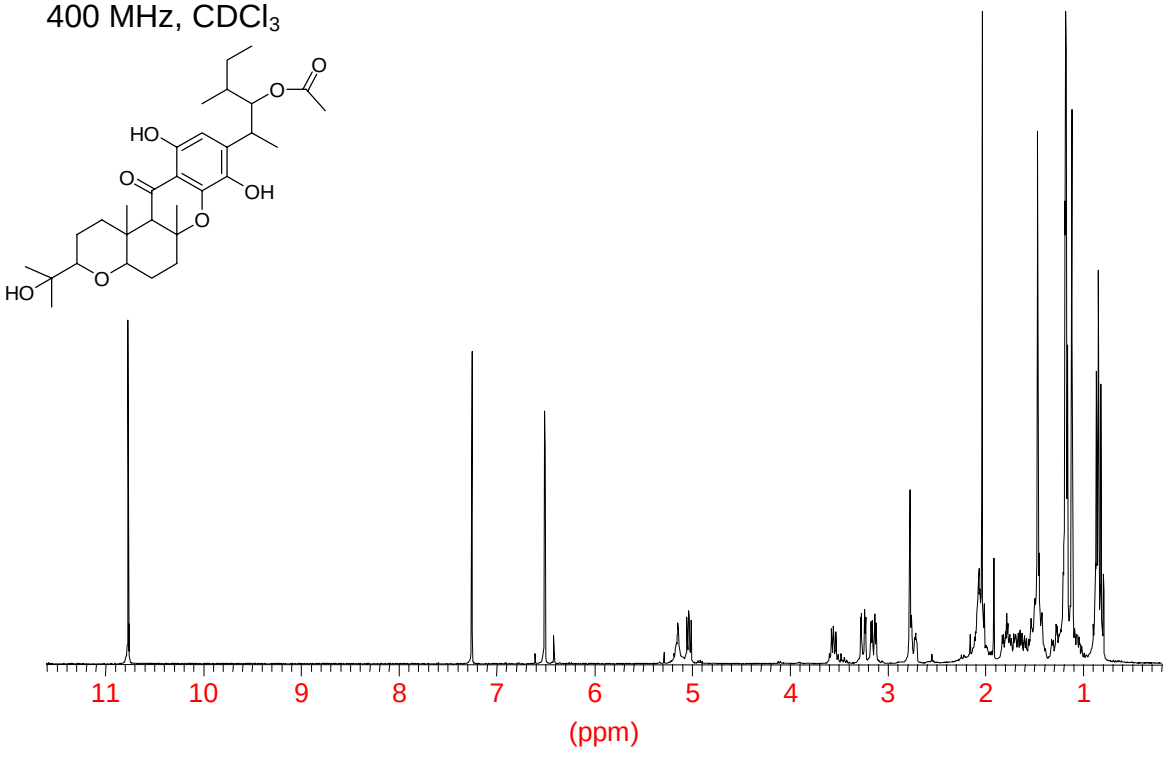
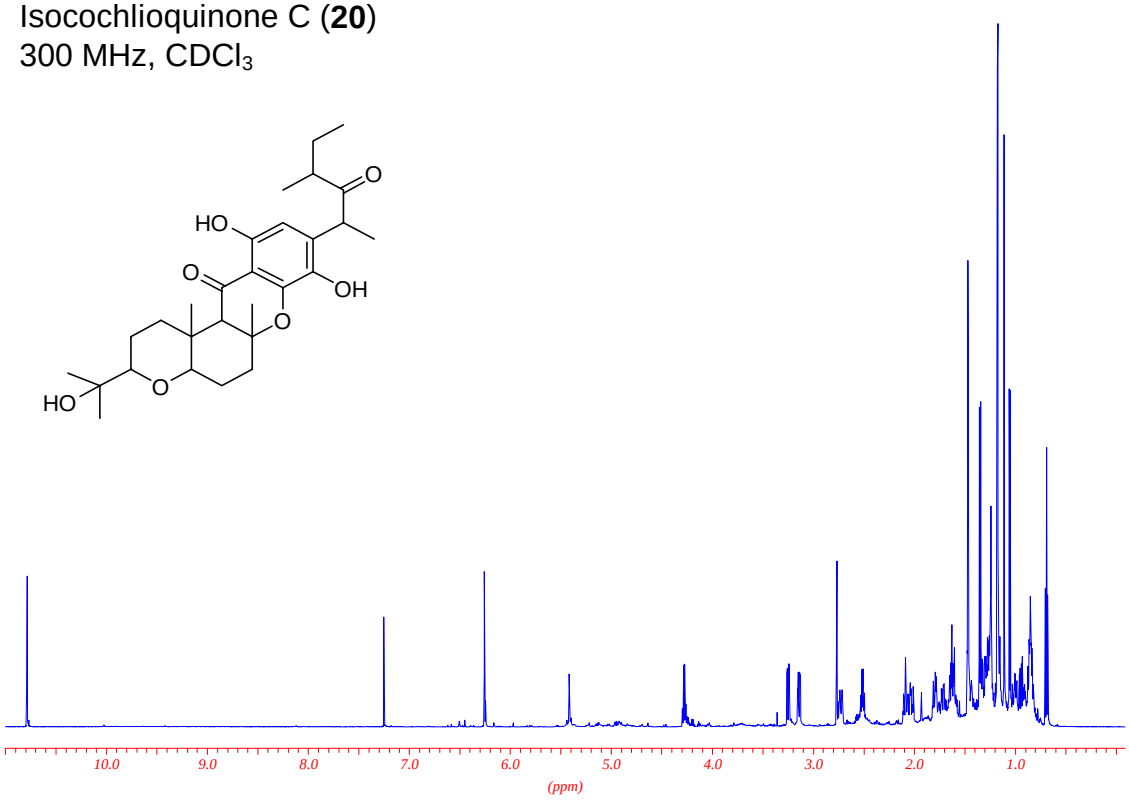


Isocochlioquinone A (**19**)
400 MHz, $CDCl_3$



Isocochlioquinone C (**20**)
300 MHz, $CDCl_3$



Cochlioquinone B (**21**)
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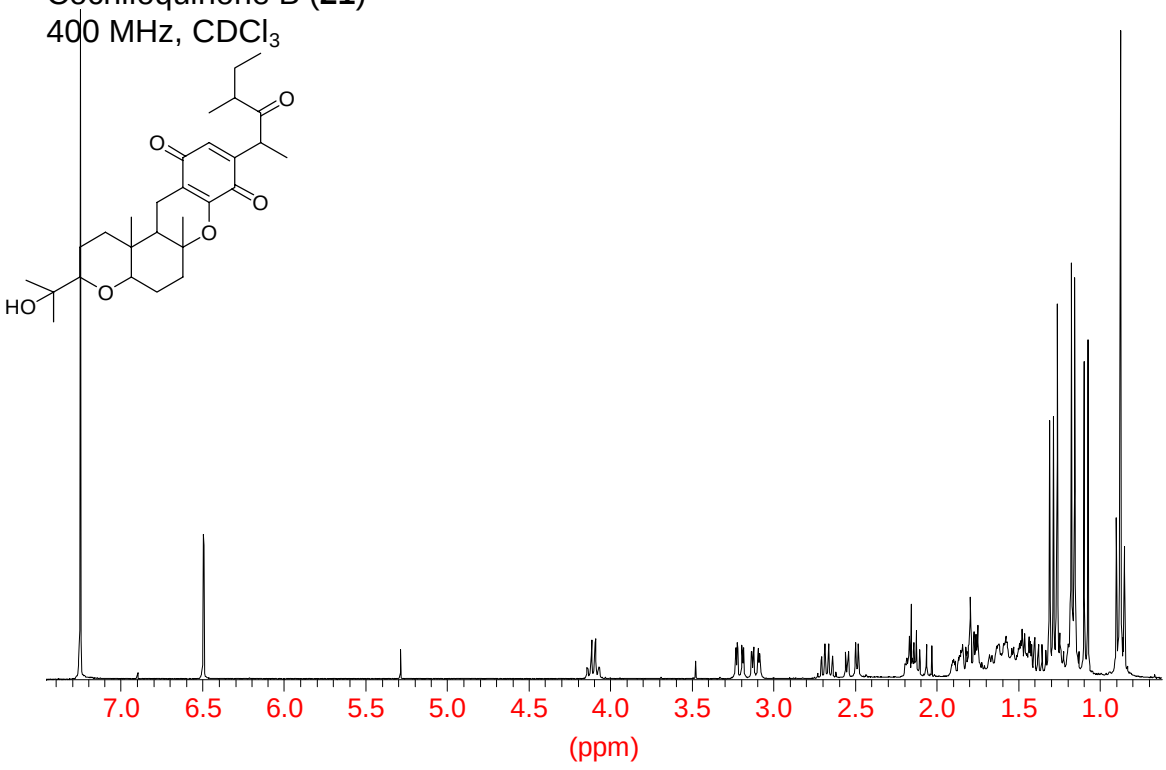

5-Hydroxyramulosin (**22**)
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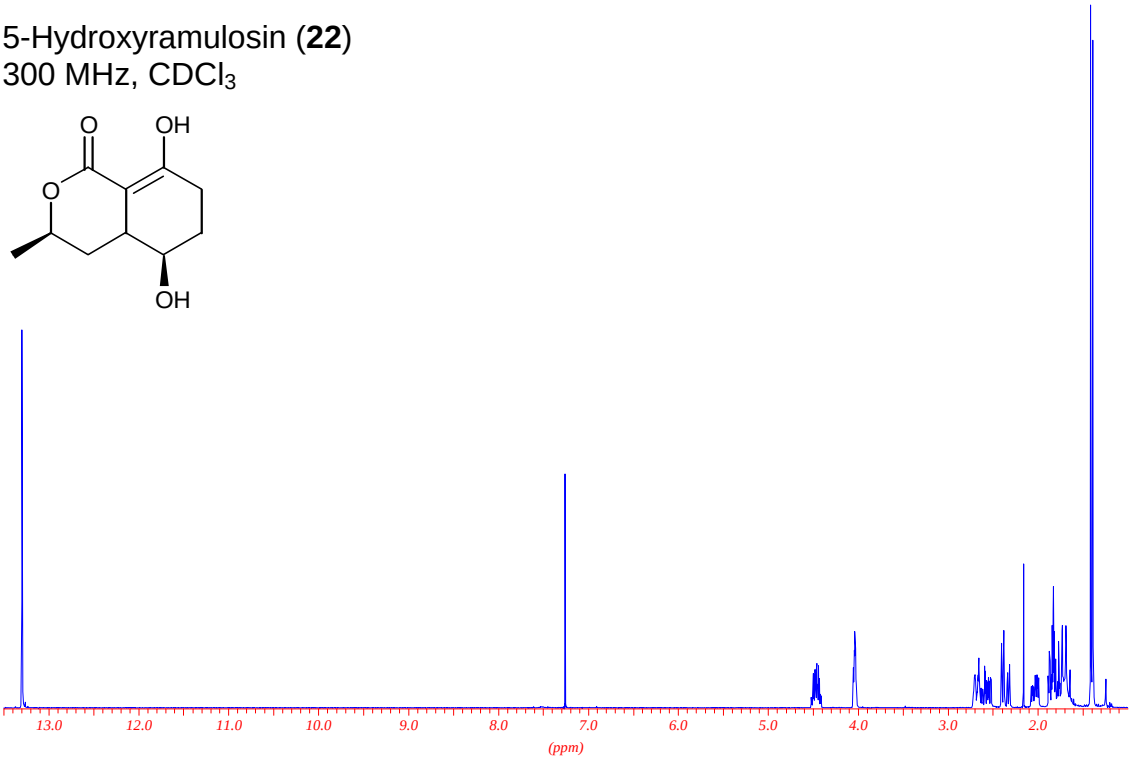
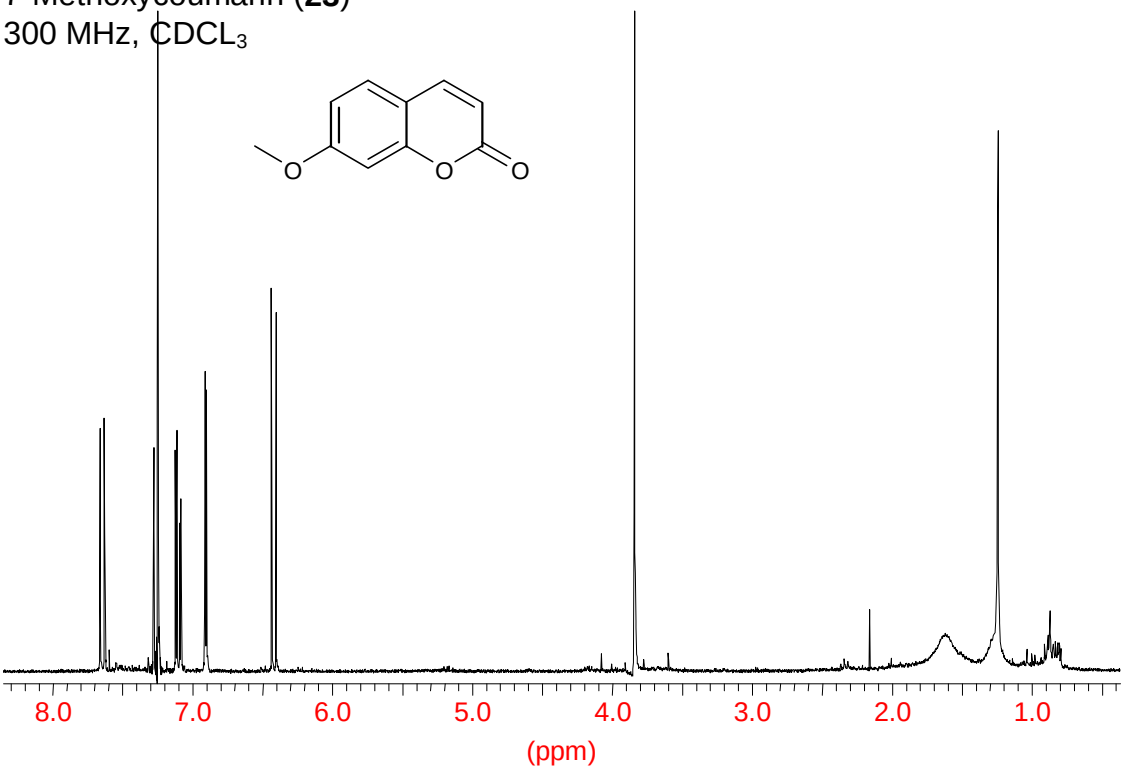
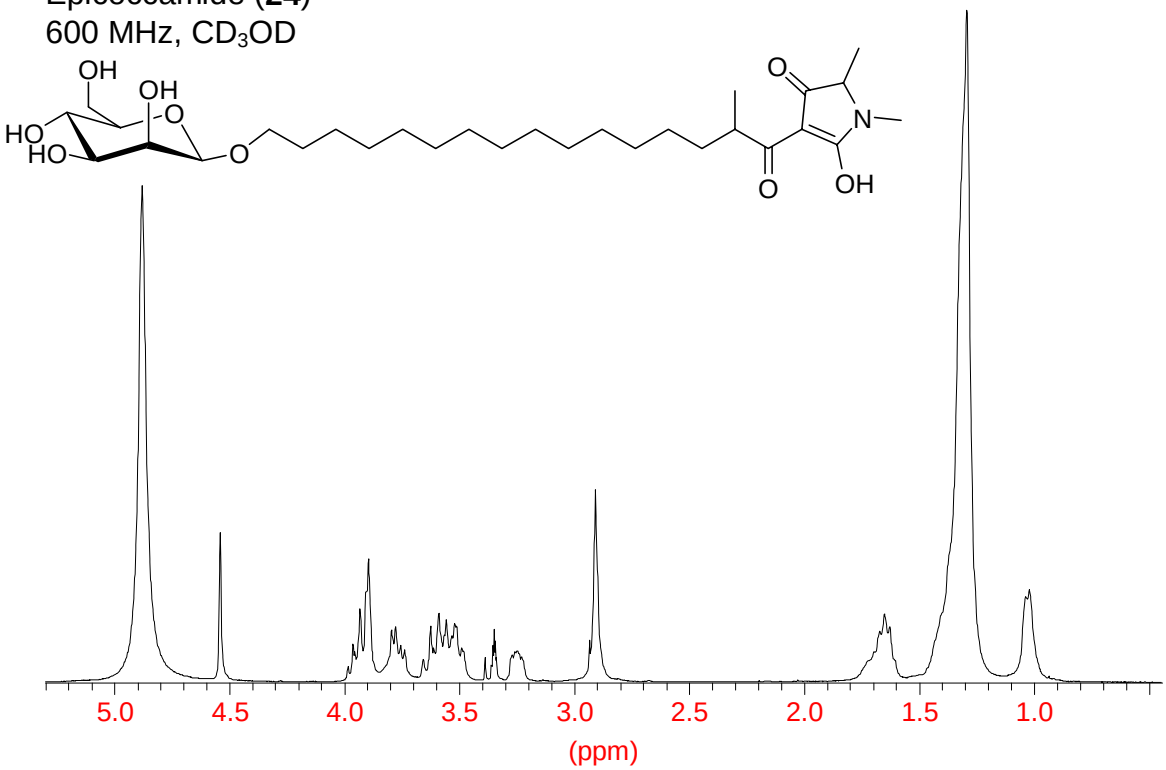


7-Methoxycoumarin (**23**)
300 MHz, $CDCL_3$



Epicoccamide (**24**)
600 MHz, CD_3OD



8.2 Results of the agar diffusion assays

Table 19: Results of the agar diffusion assays with EtOAc extracts of fungal cultures.

Fungal strain	Me-di-um	Inhibition (cm) against test organism						
		E. c.	***B. m.***	***M. v.***	***E. r.***	***F. o.***	***M. m.***	***C. f.***
M.s.	B	-	-	-	0.4	-	-	-
	MS	-	0.4	0.7	0.3	-	-	-
N1-3	Bfl	-	0.2	0.1	-	-	-	-
Arthrinium sp.	B	-	-	0.3	-	-	-	-
	MS	-	-	-	-	-	-	-
N1-5	Bfl	-	-	-	-	-	-	-
M.s.	B	-	-	-	0.4	-	-	-
	MS	-	-	-	0.3	-	-	-
N1-7	Bfl	-	0.2	0.1	-	-	-	-
M.s	B	-	-	-	0.2	-	-	-
	MS	-	-	-	0.2	-	-	-
N1-8	Bfl	-	0.2	0.2	-	-	-	-
M.s	B	-	-	0.2	0.2	-	-	-
	MS	-	-	0.1	0.2	-	-	-
N1-9	Bfl	-	0.2	0.3	0.3	-	-	-
Epicoccum sp.	B	-	-	-	-	-	-	-
	MS	-	0.2	-	0.1	-	0.1	-
N1-12	Bfl	-	-	-	0.1	-	-	-
Dendryphiella	B	-	-	0.2	-	-	-	-
salina	MS	-	-	-	-	-	-	-
N1-13	Bfl	-	-	0.2	-	-	-	-
Dendryphiella	B	-	0.1	0.2	-	-	-	-
salina	MS	-	-	0.2	-	-	-	-
N1-14	Bfl	-	0.2	0.2	-	-	-	-
Dendryphiella	B	-	0.6	0.3	0.3	0.1	0.1	-
salina	MS	0.1	0.3	0.3	-	-	-	-
N1-15	Bfl	-	-	0.1	-	-	-	-
Dendryphiella	B	-	-	0.1	0.1	-	-	-
salina	MS	-	0.2	0.2	0.3	-	-	-
N1-16	Bfl	-	-	0.1	-	-	-	-
Dendryphiella	B	-	0.2	0.2	-	-	-	-
salina	MS	-	0.2	0.3	-	-	-	-
N1-19	Bfl	-	-	0.2	-	-	-	-
Dendryphiella	B	-	0.4	0.3	0.2	-	-	-
salina	MS	-	0.4	0.3	-	-	-	-
N1-20	Bfl	-	-	-	-	-	-	-
Dendryphiella	B	-	0.1	-	0.5	-	-	-
salina	MS	-	0.2	-	0.3	-	-	-

Table 19 continued.

Fungal strain	Me-di-um	Inhibition (cm) against test organism						
		E. c.	***B. m.***	***M. v.***	***E. r.***	***F. o.***	***M. m.***	***C. f.***
N2-3	Bfl	-	0.1	0.2	0.2	-	-	-
Dendryphiella	B	-	0.6	0.6	0.3	-	-	-
salina	MS	-	0.5	0.4	0.2	-	-	-
N2-4	Bfl	-	-	-	-	-	-	-
Dendryphiella	B	-	0.5	0.3	-	-	-	-
salina	MS	-	0.5	0.4	-	-	0.2	-
N2-5	Bfl	-	0.4	0.5	-	0.1	0.2	-
M.s.	B	-	-	0.3	0.2	-	-	-
	MS	-	-	-	-	-	-	-
N2-8	Bfl	-	0.1	-	0.2	-	-	-
M.s.	B	-	0.2	0.5	0.1	-	-	-
	MS	-	0.3	0.3	0.4	-	-	-
N2-9	Bfl	-	0.3	0.1	0.3	-	-	-
Dendryphiella	B	-	0.3	0.4	-	-	-	-
salina	MS	-	0.4	0.3	-	-	-	-
N2-10	Bfl	-	0.3	0.4	-	-	0.2	-
Dendryphiella	B	-	0.3	0.2	0.3	-	0.2	-
salina	MS	-	0.4	-	0.3	-	0.1	-
N2-16	Bfl	-	-	0.2	-	-	-	-
M.s.	B	-	0.5	0.1	-	-	-	-
	MS	-	0.2	-	0.3	-	-	-
N3-1	Bfl	-	0.3	0.3	0.1	-	-	-
M.s.	B	-	-	0.2	0.2	-	-	-
	MS	-	-	-	-	-	-	-
N3-5	Bfl	-	0.1	0.1	-	-	-	-
M.s.	B	-	-	0.2	0.1	-	-	-
	MS	-	-	-	-	-	-	-
N3-6	Bfl	-	0.2	0.3	-	-	-	-
M.s.	B	-	0.1	1.7	1.6	0.7	-	-
	MS	-	0.1	2.8	2.0	1.2	-	-
N4-1	Bfl	-	0.4	1.2	0.7	1.0	-	-
Ascochyta	B	-	0.4	0.2	0.2	-	0.2	-
salicorniae	MS	-	0.1	-	-	-	-	-
N4-2	Bfl	-	0.8	0.2	0.5	-	0.7	-
Drechslera sp.	B	-	0.4	0.3	0.2	-	-	-
	MS	-	-	-	-	-	-	-
N4-6	Bfl	-	0.3	0.3	0.3	-	-	-
M.s.	B	-	0.4	0.5	0.5	-	-	-
	MS	-	0.1	-	-	-	-	-
N5-1	Bfl	-	0.3	0.3	0.5	-	-	-
Dendryphiella	B	-	0.5	0.2	0.3	-	0.2	-
salina	MS	-	0.4	-	0.3	-	0.1	-
N5-2	Bfl	-	-	0.2	-	-	-	-

Table 19 continued.

Fungal strain	Me-di-um	Inhibition (cm) against test organism						
		E. c.	***B. m.***	***M. v.***	***E. r.***	***F. o.***	***M. m.***	***C. f.***
Dendryphiella	B	-	-	-	0.2	-	-	-
salina	MS	-	0.3	-	0.1	-	-	-
N5-5	Bfl	-	-	0.2	0.2	-	-	-
M.s.	B	0.4	0.2	-	0.1	-	-	-
	MS	-	0.4	0.4	0.4	-	-	-
N6-1	Bfl	-	0.1	0.3	-	-	-	-
M.s.	B	-	0.1	0.1	-	-	-	-
	MS	-	-	0.1	-	-	-	-
N6-4	Bfl	-	-	-	-	-	-	-
M.s.	B	-	-	0.2	-	-	-	-
	MS	-	0.2	0.2	0.2	-	-	-
N6-5	Bfl	-	-	0.2	0.2	0.1	-	-
M.s.								
N6-8	Bfl	-	0.1	0.4	-	-	-	-
M.s.	B	-	-	0.2	-	-	-	-
	MS	-	0.4	0.7	-	-	-	-
N6-9	Bfl	-	0.6	0.4	0.4	-	-	-
M.s.	B	-	-	0.8	0.2	-	-	-
	MS	-	0.2	0.2	-	-	-	-
N7-2	Bfl	-	0.2	-	0.1	-	-	-
M.s.	B	-	0.4	0.6	-	-	-	-
	MS	0.6	0.4	-	0.2	-	-	-
N7-4	Bfl	0.4	0.3	0.5	0.5	0.2	0.2	-
M.s.	B	-	-	-	-	-	-	-
	MS	-	0.2	0.3	0.3	-	-	-
N7-5	Bfl	-	0.3	-	0.3	-	-	-
Epicoccum	B	-	0.3	0.1	0.5	-	0.1	-
purpurascens	MS	-	0.1	0.2	0.3	-	-	-
N7-8	Bfl	-	0.2	-	-	-	-	-
M.s.	B	-	0.1	0.2	-	-	-	-
	MS	-	-	-	0.3	-	-	-
N7-9	Bfl	-	-	0.3	-	-	-	-
M.s.	B	-	0.6	1.2	1.0	-	-	-
	MS	-	0.5	0.7	0.4	-	-	-
CUX 1 T5	Bfl	-	0.3	0.9	0.8	-	-	-
M.s.	B	-	0.7	1.2	1.5	0.2	-	-
	MS	-	0.7	0.8	0.5	0.1	-	-
CUX1T5-1	Bfl	-	-	-	-	0.1	-	-
M.s.	B	-	0.6	TI	1.2	-	-	-
	MS	-	0.1	TI	1.0	-	-	0.4
CUX1T5-2	Bfl	-	0.3	TI	1.0	-	-	0.2

Table 19 continued.

Fungal strain	Me-di-um	Inhibition (cm) against test organism						
		E. c.	***B. m.***	***M. v.***	***E. r.***	***F. o.***	***M. m.***	***C. f.***
Myrioconium sp.	B	-	0.1	1.0	0.9	-	-	-
	MS	-	0.5	1.0	1.0	-	-	-
CUX1-1-1o	Bfl	-	0.2	0.8	0.3	-	-	-
M.s.	B	-	0.2	0.2	-	0.2	-	-
	MS	-	-	0.5	-	0.1	-	-
CUX1-1-2o	Bfl	-	0.2	0.4	0.1	0.2	-	-
Phialophora sp.	B	-	0.1	-	-	-	-	-
	MS	-	0.1	-	0.2	-	-	-
CUX1-2-1-1o	Bfl	-	0.2	-	-	-	-	-
Phialophora sp.	B	-	-	0.1	0.2	-	-	-
	MS	0.1	0.2	0.2	0.7	-	-	-
CUX1-2-2o	Bfl	-	-	0.1	0.5	-	-	-
M.s.	B	-	0.3	0.5	-	-	-	-
	MS	-	-	0.2	-	-	-	-
CUX1-2-3o	Bfl	-	-	-	-	-	-	-
Myrioconium sp.	B	-	0.1	0.7	0.2	-	-	-
	MS	-	0.2	1.3	0.4	-	-	-
CUX1-3o	Bfl	-	0.1	0.5	0.3	-	-	-
Myrioconium sp.	B	-	-	-	-	-	-	-
	MS	-	-	-	-	-	-	-
CUX1-3-1	Bfl	-	-	-	-	-	-	-
Phialophora sp.	B	-	0.1	-	0.1	0.1	-	-
	MS	-	-	-	0.3	0.2	-	-
CUX1-4	Bfl	-	0.1	-	0.2	0.2	-	-
Phialophora sp.	B	-	0.1	0.3	-	-	-	-
	MS	-	0.1	0.2	-	-	-	-
CUX1-4o	Bfl	-	0.3	0.5	-	-	-	-
Myrioconium sp.	B	-	0.2	0.5	-	-	-	-
	MS	-	0.2	0.2	-	-	-	-
CUX1-5	Bfl	-	-	0.2	-	-	-	-
Phialophora sp.	B	0.2	-	-	-	-	-	-
	MS	-	-	0.1	-	-	-	-
CUX1-5-1	Bfl	-	0.2	0.2	-	-	-	-
Phialophora sp.	B	-	0.3	-	-	-	-	-
	MS	-	-	-	-	-	-	-
CUX1-5-1B	Bfl	-	-	0.1	-	-	-	-
Phialophora sp.	B	-	-	0.4	-	-	-	-
	MS	-	0.3	-	-	-	-	-
CUX1-8-1	Bfl	-	-	0.4	-	-	-	-
Phialophora sp.	B	-	0.3	0.4	-	-	-	-
	MS	-	0.3	0.3	-	-	-	-
CUX1-8-2	Bfl	-	0.3	0.5	-	-	-	-
Phialophora sp.	B	-	-	0.4	-	-	-	-

Table 19 continued.

Fungal strain	Medium	Inhibition (cm) against test organism						
		E. c.	***B. m.***	***M. v.***	***E. r.***	***F. o.***	***M. m.***	***C. f.***
	MS	-	-	-	-	-	-	-
CUX1-9-1	Bfl	-	0.3	0.7	-	-	-	-
M.s.	B	-	0.2	0.1	-	-	-	1.0
	MS	-	0.3	-	-	-	-	-
CUX1-9-2	Bfl	-	-	-	-	-	-	-
Phialophora sp.	B	-	0.2	-	0.6	-	-	-
	MS	-	-	-	-	-	-	-
CUX1-11-1	Bfl	-	0.2	-	-	-	-	-
M.s.	B	-	0.9	0.3	0.4	-	-	0.3
	MS	-	0.3	0.3	0.4	-	-	-
CUX1-19	Bfl	-	-	0.3	-	-	-	-
Geotrichum sp.	B	-	-	-	-	-	-	-
	MS	-	0.5	0.5	-	-	-	-
CUX1-22	Bfl	-	-	-	-	-	-	-
Phialophora sp.	B	-	-	0.4	0.3	-	-	0.3
	MS	-	-	0.2	-	-	-	-
CUX1-23-2-1	Bfl	-	-	0.2	-	-	-	-
Phialophora sp.	B	-	0.2	0.1	-	-	-	-
	MS	-	0.1	-	-	-	-	-
CUX1-23-2-2	Bfl	-	0.1	0.2	-	-	-	-
Zygorhynchus sp.	B	-	0.2	0.3	-	0.2	-	-
	MS	-	0.3	0.4	-	0.2	-	-
CUX2-3o	Bfl	-	0.3	0.3	-	-	-	-
Myrioconium sp.	B	-	0.1	TI	0.7	-	-	-
	MS	-	1.1	TI	1.7	-	0.2	-
CUX3-2o	Bfl	-	0.2	TI	2.0	-	-	-
Myrioconium sp.	B	-	0.1	0.2	0.2	-	-	-
	MS	-	0.2	1.2	1.1	-	-	-
CUX3-3o	Bfl	-	-	1.0	0.3	-	-	-
Myrioconium sp.	B	-	-	TI	0.5	-	-	-
	MS	-	0.3	TI	1.5	-	0.1	0.3
CUX3-6	Bfl	-	-	TI	0.5	-	-	1.7
Phialophora sp.	B	-	0.2	0.2	-	-	-	-
	MS	-	0.1	0.2	-	-	-	-
CUX3-2-1-2	Bfl	-	-	0.5	-	-	-	-
Phialophora sp.	B	-	0.3	0.7	0.7	0.2	0.4	-
	MS	-	0.4	0.8	0.6	0.4	0.5	-
CUX3-3	Bfl	-	1.7	0.9	0.2	0.2	0.3	-
Phialophora sp.	B	-	-	-	0.1	-	-	-
	MS	-	-	-	0.1	-	-	-
CUX3-4-1-1	Bfl	-	0.2	-	0.3	0.3	-	-
Phialophora sp.	B	-	0.2	0.2	0.3	-	-	-
	MS	-	0.2	0.2	-	-	-	-

Table 19 continued.

Fungal strain	Me-di-um	Inhibition (cm) against test organism						
		E. c.	***B. m.***	***M. v.***	***E. r.***	***F. o.***	***M. m.***	***C. f.***
CUX3-4-1-2	Bfl	-	-	0.2	-	-	-	-
Phialophora sp.	B	-	-	0.2	-	-	-	-
	MS	-	0.2	0.5	-	-	-	-
CUX3-6-1-1	Bfl	-	-	0.4	-	-	-	-
Phialophora sp.	B	-	0.2	-	-	-	-	-
	MS	-	0.3	-	-	-	-	-
CUX3-6-1-2	Bfl	-	0.2	-	-	-	-	-
Myrioconium sp.	B	0.2	1.6	TI	0.5	-	0.2	0.3
	MS	-	0.3	TI	1.8	-	-	0.4
CUX3-8-1	Bfl	-	0.4	TI	1.4	-	-	-
Phialophora sp.	B	-	-	-	0.3	-	-	-
	MS	0.1	0.1	-	0.1	-	-	-
CUX3-9-1	Bfl	-	0.2	0.2	-	-	-	-
Myrioconium sp.	B	-	-	TI	1.2	-	-	-
	MS	-	0.2	TI	1.7	-	-	-
CUX3-10	Bfl	-	0.3	TI	2.1	-	-	-
Phialophora sp.	B	-	0.3	-	-	-	-	-
	MS	-	0.2	0.2	-	-	-	-
CUX3-12-1-1	Bfl	-	0.2	0.2	-	-	-	-
Phialophora sp.	B	-	0.1	0.2	-	-	-	-
	MS	-	-	0.1	-	-	-	-
CUX3-12-1-2	Bfl	-	0.3	0.2	-	-	-	-
M.s.	B	-	0.2	0.9	0.7	-	-	-
	MS	-	0.6	1.0	1.0	-	0.2	-
CUX3-14	Bfl	-	-	0.6	0.3	-	-	-
M.s.	B	-	0.2	0.8	1.0	-	-	-
	MS		0.7	0.8	1.5			
CUX3-15	Bfl	-	0.1	1.0	1.0	-	-	-
Dendryphiella	B	-	0.3	0.4	0.8	-	0.4	-
salina	MS	-	0.5	0.3	-	-	0.1	-
CUX3-16	Bfl	-	0.2	0.1	-	-	-	-
M.s.	B	-	1.0	1.0	0.5	-	-	-
Fucus1	MS	-	1.3	1.3	0.9	0.3	0.4	-
M.s.	B	-	0.5	1.2	1.2	-	-	-
	MS	-	0.7	1.5	1.3	-	-	-
Fucus2	Bfl	-	0.2	0.5	0.5	-	-	-
M.s.	B	-	0.3	-	0.3	-	-	-
	MS	-	0.4	1.3	0.8	-	-	-
Fucus3	Bfl	-	0.1	0.5	0.6	-	-	-
M.s.	B	-	0.1	-	0.9	-	-	-
	MS	-	0.1	0.4	0.9	-	0.2	-
Fucus5	Bfl	-	0.1	-	0.6	-	-	-

Table 19 continued.

Fungal strain	Me-di-um	Inhibition (cm) against test organism						
		E. c.	***B. m.***	***M. v.***	***E. r.***	***F. o.***	***M. m.***	***C. f.***
Stachybotrys sp.	B	-	0.2	-	-	-	-	-
	MS	-	-	0.2	-	0.1	-	-
M1T2-2	Bfl	-	0.1	0.1	-	-	-	-
M.s.	B	-	-	0.1	-	-	-	-
	MS	-	-	0.2	-	-	-	-
M1T4	Bfl	-	-	0.2	0.2	-	-	-
M.s.	B	-	-	0.1	-	-	-	-
	MS	-	-	0.1	0.1	-	-	-
M1-1-1	Bfl	-	-	-	-	-	-	-
M.s.	B	-	-	1.5	1.2	-	0.4	1.5
	MS	-	-	1.5	0.5	-	0.2	1.5
M1-7-1	Bfl	-	-	1.0	0.9	-	0.1	1.0
M.s.	B	-	-	-	-	-	-	-
	MS	-	-	0.2	-	-	-	-
M1-8-1	Bfl	-	-	-	-	-	-	-
Emericellopsis	B	-	-	-	-	-	-	-
minima	MS	-	0.4	0.2	0.2	0.1	0.2	-
M1-10-1	Bfl	-	0.3	-	-	-	-	-
Gliomastix sp.	B	-	0.3	0.2	-	-	-	-
	MS	-	0.4	0.5	0.2	-	-	-
M1-11-1	Bfl	-	0.2	0.2	-	-	-	-
Humicola	B	-	-	1.5	0.2	0.2	-	-
fuscoatra	MS	-	-	0.2	0.2	0.2	-	-
M1-13-1-1	Bfl	-	-	1.4	-	-	-	-
M.s.	B	-	0.1	-	-	-	-	-
	MS	0.7	1.0	0.1	0.4	-	-	-
M1-13-1-2	Bfl	-	0.4	-	-	-	-	-
Humicola	B	-	0.3	1.7	-	-	-	-
fuscoatra	MS	-	-	0.1	-	-	-	-
M1-14-1	Bfl	-	-	1.4	-	-	-	-
Stachybotrys sp.	B	-	0.2	0.2	-	-	-	-
	MS	-	0.1	0.2	-	-	-	-
M1-15	Bfl	-	0.2	0.2	-	-	-	-
Stachybotrys sp.	B	-	0.2	-	-	-	-	-
	MS	-	0.2	-	-	-	-	-
M1-18	Bfl	-	0.2	-	-	-	-	-
Scolecobasidium	B	-	-	0.1	0.5	-	0.2	-
arenarium	MS	-	0.3	0.5	0.2	-	0.2	-
M3T1-1	Bfl	-	-	0.3	-	-	-	-
Alternaria sp.	B	-	0.8	0.1	0.5	-	-	-
	MS	-	0.5	0.2	0.3	-	-	-
M3T2-1	Bfl	-	0.6	0.3	0.5	-	-	-
Arthroderma sp.	B	-	-	0.3	0.5	-	-	-

Table 19 continued.

Fungal strain	Me-di-um	Inhibition (cm) against test organism						
		E. c.	***B. m.***	***M. v.***	***E. r.***	***F. o.***	***M. m.***	***C. f.***
	MS	-	-	0.3	-	-	-	-
M3T5	Bfl	-	0.2	0.3	0.5	-	-	-
Emericellopsis sp.	B	-	0.2	0.2	-	-	-	-
	MS	-	0.1	0.4	-	-	-	-
M4T3	Bfl	-	0.1	0.2	-	-	-	-
Alternaria sp.	B	-	0.3	0.2	1.2	-	0.3	-
	MS	-	0.3	-	1.0	-	0.5	-
M4-20-1	Bfl	-	0.2	-	0.3	-	-	-
Alternaria sp.	B	-	0.2	0.1	0.3	-	-	0.2
	MS	-	-	-	-	-	-	-
M5T1-1	Bfl	-	-	-	0.1	-	-	-
Alternaria sp.	B	-	0.8	0.1	0.2	0.2	0.1	-
	MS	-	0.3	0.2	0.4	0.2	0.2	0.3
M5T1-2	Bfl	-	-	0.1	0.2	0.1	-	-
M.s.	B	-	0.3	0.1	0.4	0.2	-	-
	MS	-	0.2	0.1	0.2	0.1	-	-
M5T1-3	Bfl	-	-	-	-	-	-	-
Drechslera	B	-	0.3	0.8	0.6	-	0.2	0.4
dematioidea	MS	-	-	0.3	0.3	-	-	0.5
M5T2-1	Bfl	-	0.2	0.3	0.2	-	-	-
Alternaria sp.	B	-	0.2	0.3	0.3	-	-	-
	MS	-	0.2	0.1	-	-	-	-
M5T2-2	Bfl	-	-	-	-	-	-	-
Alternaria sp.	B	-	-	-	0.2	-	-	0.3
	MS	-	0.3	0.2	0.3	-	-	0.3
M5T2-3	Bfl	-	-	-	-	-	-	0.3
M.s.	B	-	0.8	0.1	0.2	0.2	0.1	-
	MS	-	0.7	0.1	0.2	-	-	-
M5T3-1	Bfl	-	-	-	-	-	-	-
Alternaria sp.	B	-	-	0.2	0.3	-	-	-
	MS	-	0.3	-	0.4	-	-	-
M5T3-2	Bfl	-	-	-	-	-	-	-
M.s.	B	-	0.3	0.2	-	-	-	-
	MS	-	0.3	0.2	-	-	-	-
M5T3-3	Bfl	-	-	-	-	-	-	-
Chaetomium sp.	B	-	0.8	0.5	0.3	-	-	-
	MS	-	0.8	0.7	0.5	-	0.2	-
M5-5-1	Bfl	-	1.6	0.3	0.2	-	-	-
M.s.	B	-	0.1	-	0.2	-	-	-
	MS	-	0.1	-	0.4	-	-	-
M6T2	Bfl	-	-	-	-	-	-	-
M.s.	B	-	0.2	0.2	-	-	-	-
	MS	-	0.4	0.2	-	-	-	-

Table 19 continued.

Fungal strain	Me-di-um	Inhibition (cm) against test organism						
		E. c.	***B. m.***	***M. v.***	***E. r.***	***F. o.***	***M. m.***	***C. f.***
M6T4-1	Bfl	-	0.3	-	-	-	-	-
Chaetomium sp.	B	-	0.3	0.2	-	-	-	-
	MS	-	-	0.2	-	-	-	-
M6-3-1	Bfl	-	-	-	-	-	-	-
M.s.	B	-	0.1	-	-	-	-	-
	MS	-	0.2	-	-	-	-	-
M6-3-4	Bfl	-	-	-	-	-	-	-
M.s.	B	-	0.2	-	-	-	-	-
	MS	-	0.2	-	-	-	-	-
M6-4-1	Bfl	-	-	-	-	-	-	-
Alternaria sp.	B	-	-	0.1	0.3	-	-	-
	MS	-	0.3	0.3	-	-	-	0.5
M7T1-1	Bfl	-	-	0.2	0.1	-	-	-
Chaetomium sp.	B	-	0.2	0.3	0.2	-	-	-
	MS	-	-	0.2	-	-	-	-
M7T1-2	Bfl	-	-	0.3	0.2	-	-	-
M.s.	B	-	0.2	-	-	-	-	-
	MS	-	0.2	-	0.4	-	-	-
M7-5-1	Bfl	-	-	-	-	-	-	-
M.s.	B	-	0.3	-	-	-	-	-
	MS	-	0.3	-	-	-	-	-
M7-5-6	Bfl	-	-	-	-	-	-	-
Xylaria sp.	B	-	-	0.2	-	-	-	-
	MS	-	-	0.3	0.3	-	0.3	-
M7-6-1	Bfl	-	-	-	-	-	-	-
Stemphylium sp.	B	1.0	0.5	0.1	0.3	-	0.1	-
	MS	-	0.3	0.4	-	-	-	-
M7-8-1	Bfl	-	-	-	0.2	-	0.1	-
M.s.	B	-	-	-	-	-	-	-
	MS	-	-	-	-	-	-	-
M7-9-1	Bfl	-	0.1	-	-	-	-	-
Alternaria sp.	B	-	0.5	0.1	0.8	-	-	0.2
	MS	-	0.1	-	-	-	-	-
M7-10-1	Bfl	-	0.2	-	0.3	-	-	-
Alternaria sp.	B	-	0.2	0.5	1.0	-	0.1	-
	MS	-	0.4	0.2	0.6	-	0.1	-
M7-11-1	Bfl	-	0.2	0.1	0.5	-	-	-
Chaetomium sp.	B	-	0.5	-	0.3	-	0.2	0.1
	MS	-	0.2	-	0.3	-	0.2	-
M7-13-1	Bfl	-	0.1	-	0.2	-	-	-
Alternaria sp.	B	-	0.5	-	0.3	-	0.2	0.1
	MS	-	0.2	-	0.3	-	0.2	-
M7-19	Bfl	-	0.1	-	0.2	-	-	-

Table 19 continued.

Fungal strain	Me-di-um	Inhibition (cm) against test organism						
		E. c.	***B. m.***	***M. v.***	***E. r.***	***F. o.***	***M. m.***	***C. f.***
Stachybotrys sp.	B	-	0.2	0.1	-	-	-	-
	MS	-	0.3	-	-	-	-	-
M8T1-1	Bfl	-	0.1	-	-	-	-	-
Paecilomyces sp.	B	-	0.8	1.1	1.0	-	0.2	-
	MS	-	1.0	1.3	1.1	-	0.3	-
M8T1-2	Bfl	-	0.6	1.2	1.2	-	0.4	-
Stachybotrys sp.	B	-	0.3	-	-	-	-	-
	MS	-	0.1	-	-	-	-	-
M8T2	Bfl	-	-	0.1	-	-	-	-
M.s.	B	-	0.1	0.2	-	-	-	-
	MS	-	0.1	0.7	-	-	-	0.2
M8-3-1	Bfl	-	-	1.1	0.2	0.2	0.3	0.6
Chaetomium sp.	B	-	-	0.3	0.7	-	-	-
	MS	-	-	-	0.8	-	-	-
M8-11-1	Bfl	-	-	-	0.7	-	-	-
M.s.	B	-	-	-	-	-	-	-
	MS	-	-	-	-	-	-	-
M8-20-1	Bfl	-	-	-	-	-	-	-
Stachybotrys sp.	B	-	0.1	-	0.2	-	-	-
	MS	-	0.3	0.2	0.1	-	-	-
G1-9-1; 40 sec	Bfl	-	0.1	0.2	-	-	-	-
Sporormiella sp.	B	-	-	0.3	0.9	-	0.3	1.2
	MS	-	-	0.2	1.0	-	0.8	1.5
G2-1; 30 sec	Bfl	-	-	0.2	0.9	-	0.3	1.3
M.s.	B	-	-	-	-	-	-	-
	MS	-	-	1.0	-	-	-	-
G2-4-1	Bfl	-	-	1.3	1.0	0.2	-	-
Phoma sp.	B	-	0.2	-	-	-	-	-
	MS	-	0.2	0.2	-	-	-	-
G2-5; 90 sec	Bfl	-	0.3	0.2	0.2	-	-	-
Phoma sp.	B	-	-	0.3	-	-	-	-
	MS	-	-	0.3	-	-	-	-
G2-10; 30 sec	Bfl	-	-	-	-	-	-	-
Nigrospora sp.	B	-	-	0.3	0.5	-	-	-
	MS	-	-	0.3	0.2	-	-	-
G2-10; 60 sec	Bfl	-	-	-	-	-	-	-
M.s.	B	-	-	-	-	-	-	-
	MS	-	-	-	-	-	-	-
G2-13; 90 sec	Bfl	-	-	0.2	-	-	-	-
Stachybotrys sp.	B	-	0.1	0.4	0.4	-	-	-
	MS	-	-	0.2	0.2	-	-	-
G2-16; 60 sec	Bfl	-	-	-	-	-	-	-

Table 19 continued.

Fungal strain	Me-di-um	Inhibition (cm) against test organism						
		E. c.	***B. m.***	***M. v.***	***E. r.***	***F. o.***	***M. m.***	***C. f.***
M.s.	B	-	0.4	0.3	-	-	-	-
	MS	-	0.3	0.1	-	-	-	-
AZ1-1	Bfl	-	0.3	0.2	-	-	-	-
Chaetomella sp.	B	-	-	-	-	-	-	-
	MS	-	-	0.3	-	-	-	-
AZ3-2-1	Bfl	-	-	0.2	-	-	-	-
M.s.	B	-	-	-	-	-	-	-
	MS	-	0.4	0.4	-	-	-	-
AZ3-3	Bfl	-	-	-	-	-	-	-
Phoma sp.	B	-	-	-	-	-	-	-
	MS	-	0.2	0.2	-	-	-	-
AZ3-4	Bfl	-	-	-	-	-	-	-
Phoma sp.	B	-	0.5	0.2	-	-	-	-
	MS	-	0.6	0.4	-	-	-	-
AZ3-4-1	Bfl	-	-	0.1	-	-	-	-
Phoma sp.	B	-	-	-	-	-	-	-
	MS	-	0.3	0.2	-	-	0.2	0.3
AZ3-4-2	Bfl	-	0.2	0.1	-	-	-	-
Phoma sp.	B	-	-	-	-	-	-	-
	MS	-	0.3	0.4	0.2	-	-	-
AZ3-4-3	Bfl	-	-	-	-	-	-	-
M.s.	B	-	0.4	0.8	0.2	-	0.1	-
	MS	-	0.4	-	-	-	-	-
AZ3-4	Bfl	-	0.4	0.8	0.4	-	0.1	-
Chaetomium sp.	B	-	0.3	0.2	-	-	-	-
	MS	-	0.1	0.2	0.1	-	-	-
AZ3-10	Bfl	-	0.7	0.7	0.8	-	0.7	-
Phoma sp.	B	-	-	0.1	-	-	-	-
	MS	-	0.3	0.2	-	-	-	0.2
AZ3-18	Bfl	-	0.1	0.1	-	-	-	-
Preussia sp.	B	-	-	-	-	-	-	-
	MS	-	0.4	0.2	-	-	-	-
AZ4-12-1	Bfl	-	0.3	0.2	-	-	-	-
Pestalotiopsis sp.	B	-	-	-	-	-	-	-
	MS	-	-	0.3	0.3	-	-	-
AZ5T4	Bfl	-	-	0.1	-	-	-	-
M.s.	B	-	-	-	-	-	-	-
	MS	-	0.5	0.4	0.3	-	-	-
AZ5-2	Bfl	-	-	-	-	-	-	-
Cylindrocarpon sp.	B	-	-	1.1	-	-	-	-
	MS	-	0.2	2.0	0.2	-	-	-
AZ5-10-1	Bfl	-	0.3	0.7	-	-	-	-

Table 19 continued.

Fungal strain	Me-di-um	Inhibition (cm) against test organism						
		E. c.	***B. m.***	***M. v.***	***E. r.***	***F. o.***	***M. m.***	***C. f.***
M.s.	B	-	0.1	0.3	-	-	-	-
	MS	-	0.3	0.1	-	-	-	-
AZ5-18-1	Bfl	-	-	0.1	-	-	-	-
Chaetomium sp.	B	-	1.0	0.3	-	-	-	-
	MS	-	1.5	0.6	0.2	0.3	-	-
AZ6-1-1	Bfl	-	1.2	0.2	0.3	0.1	-	-
M.s.	B	-	1.0	0.8	0.3	-	-	-
	MS	-	0.9	1.0	0.7	-	-	-
AZ6-3	Bfl	-	1.0	-	0.6	-	-	-
Gliomastix sp.	B	0.2	0.3	0.2	0.3	-	-	-
	MS	-	0.3	0.2	0.3	-	-	-
AZ6-5-1	Bfl	-	0.5	0.2	0.2	-	-	0.5
Chaetomium sp.	B	-	-	-	0.1	-	-	-
	MS	-	0.2	-	0.2	-	-	-
AZ6-10-1	Bfl	-	-	-	0.2	-	-	-
Scopulariopsis sp.	B	-	-	0.4	-	-	-	-
	MS	-	-	0.3	-	-	-	-
AZ7-13-1	Bfl	-	-	0.2	-	-	-	-
Scopulariopsis sp.	B	-	0.1	0.2	0.1	-	-	-
	MS	-	-	1.0	0.2	-	-	-
AZ7-13-2	Bfl	-	-	0.3	0.3	0.2	-	-
Scopulariopsis sp.	B	-	-	1.3	0.3	-	-	-
	MS	-	-	0.9	0.2	0.4	-	-
AZ7-13-3	Bfl	-	-	0.4	-	-	-	-
Wardomyces	B	-	0.4	0.2	-	-	-	-
inflatus	MS	-	0.3	0.3	-	-	-	-
AZ7-13-4	Bfl	-	0.2	0.2	-	-	-	-
M.s.	B	-	-	-	-	-	-	-
	MS	-	0.2	0.2	-	-	-	0.2
AZ8-6-1	Bfl	-	0.1	-	-	-	-	-
Geotrichum sp.	B	-	-	0.1	0.1	-	-	-
	MS	-	-	0.3	-	-	-	0.4
AZ9-12-1	Bfl	-	0.4	-	-	-	-	-
Phoma sp.	B	-	0.2	0.3	-	-	-	-
	MS	-	0.1	0.2	-	-	-	-
AZ10-20-1-1	Bfl	-	-	0.2	-	-	-	-
Phoma sp.	B	-	0.2	0.2	0.2	0.1	-	0.1
	MS	-	0.2	-	0.2	-	-	-
AZ10-20-1-2	Bfl	-	-	-	0.2	-	-	-
M.s.	B	-	-	0.2	0.2	-	-	-
	MS	-	-	0.2	0.2	0.2	-	-
AZ11-4-1	Bfl	-	-	0.2	0.2	-	-	-
M.s.	B	-	-	0.2	0.3	-	-	-

Table 19 continued.

Fungal strain	Me-di-um	Inhibition (cm) against test organism						
		E. c.	***B. m.***	***M. v.***	***E. r.***	***F. o.***	***M. m.***	***C. f.***
	MS	-	-	0.2	0.3	-	-	-
AZ11-9-1	Bfl	-	-	-	-	-	-	-
M.s.	B	-	-	-	-	-	-	-
	MS	-	-	-	-	-	-	-
AZ11-18-1	Bfl	-	-	0.1	-	-	-	-
M.s.	B	-	-	0.4	0.4	-	-	-
	MS	-	-	0.2	0.2	-	-	-
AZ11-21-2	Bfl	-	-	-	-	-	-	-
M.s.	B	-	0.5	0.1	-	-	-	-
	MS	-	-	0.2	-	-	-	-
AZ12T3	Bfl	-	-	0.1	-	-	-	-
Geomyces sp.	B	-	0.3	0.4	0.2	-	-	-
	MS	-	0.2	0.2	-	-	-	-
AZ12-10-1	Bfl	-	0.3	0.4	-	-	-	-
Phoma tropica	B	-	0.3	0.3	0.2	-	-	0.3
	MS	-	0.9	0.3	-	-	-	-
AZ14-4	Bfl	-	0.2	0.2	-	-	-	-
M.s.	B	-	-	-	-	-	-	-
	MS	-	-	-	-	-	-	-
AZ14-10	Bfl	-	-	-	-	-	-	-
M.s.	B	-	0.3	-	0.1	-	-	-
	MS	-	0.3	-	0.2	-	-	0.1
AZ14-17-1	Bfl	-	0.2	-	0.2	-	-	-
M.s.	B	-	0.4	-	0.5	-	-	-
	MS	-	-	-	-	-	0.2	-
SY1-5-1	Bfl	-	-	-	-	-	-	-
M.s.	B	-	0.2	0.1	-	0.2	-	-
	MS	-	-	-	0.1	-	0.6	-
SY1-7-1	Bfl	-	-	-	-	-	-	-
M.s.	B	-	-	0.2	0.4	-	-	-
	MS	-	-	-	-	-	-	-
SY1-8-1	Bfl	-	-	0.2	0.2	-	-	-
M.s.	B	-	-	0.2	-	-	-	-
	MS	-	-	0.1	-	-	-	-
SY1-13-1	Bfl	-	-	0.1	-	-	-	-
M.s.	B	-	-	-	-	-	-	-
	MS	-	0.3	-	-	-	-	-
SY1-18-1	Bfl	-	-	-	-	-	-	-
Phialophora sp.	B	-	0.2	-	-	-	-	-
	MS	-	0.1	0.1	-	-	-	0.5
SY2Abkl1	Bfl	-	0.3	0.2	-	-	-	0.2
Phialophora sp.	B	-	0.1	0.3	0.3	-	-	-

Table 19 continued.

Fungal strain	Me-di-um	Inhibition (cm) against test organism						
		E. c.	***B. m.***	***M. v.***	***E. r.***	***F. o.***	***M. m.***	***C. f.***
	MS	-	0.1	0.2	0.3	-	-	-
SY2T1-1	Bfl	-	0.3	0.2	0.4	-	-	-
Phoma sp.	B	-	-	0.1	-	-	-	-
	MS	-	-	0.1	-	-	-	-
SY2T1-2	Bfl	-	0.1	0.3	-	-	-	-
Phialophora sp.	B	-	0.1	-	-	-	-	0.3
	MS	-	0.3	0.2	-	-	-	0.2
SY2T1-3	Bfl	-	0.3	0.2	-	-	-	0.2
Phoma sp.	B	-	0.7	0.1	-	-	-	-
	MS	-	-	0.2	-	-	-	-
SY2T1-4	Bfl	-	0.4	0.4	-	-	-	-
Phialophora sp.	B	-	0.2	0.2	0.2	-	-	-
	MS	-	0.1	0.2	-	-	-	-
SY2T2-1	Bfl	-	0.2	0.1	0.3	-	-	-
Phialophora sp.	B	-	0.2	-	-	-	-	0.2
	MS	-	0.1	-	-	-	-	0.4
SY2T2-2	Bfl	-	0.2	0.3	-	-	-	0.2
Phialophora sp.	B	0.1	0.1	0.1	-	-	-	-
	MS	-	0.1	0.2	-	-	-	-
SY2T2-3	Bfl	-	0.4	0.2	-	-	-	-
Phialophora sp.	B	-	-	0.2	-	-	-	-
	MS	-	-	-	-	-	-	-
SY2T2-4	Bfl	-	-	-	-	-	-	0.3
Phialophora sp.	B	-	0.3	-	-	-	-	0.3
	MS	-	0.1	-	-	-	-	-
SY2T2-5	Bfl	-	0.8	0.3	-	-	-	-
Phialophora sp.	B	-	0.2	0.3	-	-	-	0.2
	MS	-	0.2	-	-	-	-	-
SY2-1-1	Bfl	-	0.2	0.3	-	-	-	0.2
Phialophora sp.	B	-	0.2	-	-	-	-	-
	MS	-	0.3	-	-	-	-	-
SY2-2-1	Bfl	-	0.3	-	-	-	-	-
Phialophora sp.	B	-	-	-	-	-	-	-
	MS	-	0.3	-	-	-	-	-
SY2-3-1	Bfl	-	-	-	-	-	-	-
Phialophora sp.	B	-	-	0.1	-	-	-	-
	MS	-	0.2	-	-	-	-	-
SY2-6-1	Bfl	-	0.1	-	-	-	-	-
Phialophora sp.	B	-	-	0.2	-	-	-	-
	MS	-	0.2	-	-	-	-	-
SY2-16-1	Bfl	-	-	-	-	-	-	-
Phialophora sp.	B	-	0.1	-	-	-	-	-
	MS	-	0.1	0.2	-	-	-	-

Table 19 continued.

Fungal strain	Me-di-um	Inhibition (cm) against test organism						
		E. c.	***B. m.***	***M. v.***	***E. r.***	***F. o.***	***M. m.***	***C. f.***
SY2-18-1	Bfl	-	0.2	0.1	-	-	-	-
Wardomyces	B	-	0.1	-	-	-	-	-
inflatus	MS	-	-	-	-	-	-	-
SY3-1-1	Bfl	-	-	-	-	-	-	-
Wardomyces	B	-	0.2	0.4	-	-	-	-
inflatus	MS	-	-	-	-	-	-	-
SY3-4-1	Bfl	-	-	-	-	-	-	-
Phialophora sp.	B	-	0.2	-	-	-	-	-
	MS	-	-	-	-	-	-	-
SY3-5-1	Bfl	-	0.2	-	-	-	-	-
Phialophora sp.	B	-	-	0.3	-	-	-	-
	MS	-	-	0.2	-	-	-	-
SY3-11-1	Bfl	-	-	0.3	0.2	-	-	-
Wardomyces	B	-	-	-	-	-	-	-
anomalus	MS	-	-	-	-	-	-	-
SY3-15-1	Bfl	-	0.2	0.1	-	-	-	-
Wardomyces	B	-	0.1	-	-	-	-	-
anomalus	MS	-	-	-	-	-	-	-
SY3-16-1	Bfl	-	-	-	-	-	-	-
Wardomyces	B	-	-	-	-	-	-	-
anomalus	MS	-	-	-	-	-	-	-
SY3-18-1	Bfl	-	0.2	-	-	-	-	-
Phialophora sp.	B	-	0.2	-	-	-	-	-
	MS	-	0.2	-	-	-	-	-
SY3-20-1	Bfl	-	-	0.4	-	-	-	-
M.s.	B	-	0.5	0.8	0.4	0.5	0.8	-
	MS	-	0.5	0.9	-	0.6	1.2	-
SY4T5-1	Bfl	-	0.5	0.9	-	0.3	0.8	-
M.s.	B	-	0.3	0.2	-	-	-	-
	MS	-	0.4	-	-	-	-	-
SY4-19-1	Bfl	-	-	-	-	-	-	-
M.s.	B	-	-	0.2	-	-	-	0.2
	MS	-	-	-	0.2	-	-	-
WH2T3-1	Bfl	-	-	0.1	-	-	-	0.2
Bispora	B	-	-	0.3	0.3	-	-	-
betulina	MS	-	0.2	0.2	-	-	-	-
WH2T3-2-1	Bfl	-	-	0.1	-	-	-	-
Paecilomyces sp.	B	-	0.1	0.2	-	-	-	-
	MS	-	0.2	0.2	-	-	-	-
WH2T3-2-2	Bfl	-	-	-	-	-	-	-
M.s.	B	-	-	0.2	0.2	-	-	-
	MS	-	-	0.2	0.1	-	-	-

Table 19 continued.

Fungal strain	Me-di-um	Inhibition (cm) against test organism						
		E. c.	***B. m.***	***M. v.***	***E. r.***	***F. o.***	***M. m.***	***C. f.***
WH2T3-3	Bfl	-	-	0.2	0.1	-	-	-
Sporormiella sp.	B	-	-	0.2	-	-	-	-
	MS	-	-	-	-	-	-	-
WH2T4-1	Bfl	-	0.2	-	-	-	-	-
M.s.	B	-	0.2	0.2	-	-	-	-
	MS	-	0.4	0.6	0.3	-	-	-
WH4T2-1B	Bfl	-	0.4	0.4	-	-	0.2	-
Phialophora sp.	B	-	-	0.2	-	-	-	-
	MS	-	-	0.8	0.3	-	0.2	-
WH4T2-3	Bfl	-	0.2	1.2	0.4	-	-	-
M.s.	B	-	-	-	-	-	0.2	-
	MS	-	-	-	-	-	-	-
WH4-1-1	Bfl	-	-	-	-	-	-	-
Fusarium sp.	B	-	0.6	0.5	0.5	-	0.3	-
	MS	-	0.4	-	-	-	-	-
WH4-3-1	Bfl	-	0.5	0.5	0.3	-	0.2	-
Corollospora sp.	B	-	-	0.3	0.2	-	-	-
	MS	-	0.3	0.5	-	-	-	-
S1-1	Bfl	-	-	0.5	0.4	-	-	-
Corollospora sp.	B	-	-	-	-	-	-	-
	MS	-	-	1.3	0.3	-	-	-
S1-2	Bfl	-	-	-	0.3	-	-	-
M.s.	B	-	-	-	0.2	-	-	-
	MS	-	0.2	0.3	0.3	-	-	-
S1-3	Bfl	-	-	-	-	-	-	-
Corollospora sp.	B	-	-	0.3	0.2	-	-	-
	MS	-	-	-	-	-	-	-
S2-1	Bfl	-	-	0.3	0.2	-	-	-
Corollospora sp.	B	-	-	0.2	-	-	-	-
	MS	-	0.2	0.6	0.3	-	-	-
S2-2	Bfl	-	-	0.3	0.2	-	-	-
Corollospora sp.	B	-	-	-	-	-	-	-
	MS	-	2.0	0.9	-	-	-	-
S2-3	Bfl	-	-	-	0.2	-	-	-
M. s.	B	-	-	-	0.2	-	-	-
	MS	-	-	-	0.2	-	-	-
S2-4	Bfl	-	0.4	0.2	0.3	-	-	-
M. s.	B	-	0.5	1.2	0.2	-	-	-
	MS	-	0.5	0.4	-	-	-	-
S2-6	Bfl	-	0.2	-	-	-	-	-
Fusarium sp.	B	-	0.9	1.0	0.5	0.2	0.4	-
	MS	-	0.9	1.0	0.6	-	0.4	-

Table 19 continued.

Fungal strain	Me-di-um	Inhibition (cm) against test organism						
		E. c.	***B. m.***	***M. v.***	***E. r.***	***F. o.***	***M. m.***	***C. f.***
S2-7	Bfl	-	0.1	0.1	-	-	-	-
M. s.	B	-	0.5	0.8	0.2	-	-	-
	MS	-	0.7	1.2	0.2	-	-	-
S2-8	Bfl	-	0.1	0.1	0.2	-	-	-
Geotrichum sp.	B	-	-	0.1	-	-	-	-
	MS	-	0.5	0.4	-	-	-	-
S2-9	Bfl	-	-	-	-	-	-	-
Aureobasidium sp.	B	-	0.5	0.6	-	-	-	-
	MS	-	0.8	0.3	0.2	0.5	-	-
S2-10	Bfl	-	-	-	-	-	-	-
Stemphyllium sp.	B	-	0.4	-	-	-	-	-
	MS	-	0.1	0.4	-	-	-	-
S2-11	Bfl	-	0.1	0.5	-	-	-	-
M. s.	B	-	0.6	0.3	1.3	-	-	-
	MS	-	0.8	0.3	0.2	-	-	-
S2-13	Bfl	-	0.1	-	0.2	-	-	-
M. s.	B	-	-	0.7	0.3	-	-	-
	MS	-	-	-	-	-	-	-
S5-1	Bfl	-	-	0.1	0.2	-	-	-
Geomyces	B	-	0.3	-	-	-	-	-
pannorum	MS	-	-	-	-	-	-	-
S5-2-1	Bfl	-	-	-	-	-	-	-
Chrysosporium sp.	B	-	-	0.1	0.1	-	-	-
	MS	-	-	0.2	0.3	0.2	-	-
S5-2-2	Bfl	-	-	0.2	-	-	-	-
M. s.	B	-	-	0.2	-	-	-	-
	MS	-	-	0.2	-	-	0.1	-
S5-3	Bfl	-	0.1	0.1	0.4	-	-	-
Chaetomium sp.	B	-	-	0.3	0.3	-	0.1	-
	MS	-	-	-	-	-	-	-
S5-4	Bfl	-	-	-	1.0	-	-	-
Geotrichum sp.	B	-	-	-	-	-	-	-
	MS	-	0.4	0.4	-	-	-	-
S5-6	Bfl	-	-	-	-	-	-	-
„Ascomycet“	B	-	0.1	0.2	0.2	-	-	-
	MS	-	-	0.2	0.2	-	-	-
OS1T1-2	Bfl	-	-	-	0.3	-	-	-
Scolecobasidium	B	-	-	-	-	-	-	-
arenarium	MS	-	-	-	-	-	-	-
OS1T3-1	Bfl	-	-	0.1	-	-	-	-
Phoma sp.	B	-	0.2	-	-	-	-	-
	MS	-	0.1	0.2	-	-	-	-

Table 19 continued.

Fungal strain	Me-di-um	Inhibition (cm) against test organism						
		E. c.	***B. m.***	***M. v.***	***E. r.***	***F. o.***	***M. m.***	***C. f.***
OS1T3-3	Bfl	-	-	0.2	0.3	-	-	-
M. s.	B	-	0.2	-	-	-	-	-
	MS	-	-	-	-	-	-	-
OST3-4	Bfl	-	0.2	-	-	-	-	-
M. s.	B	-	0.5	0.2	0.2	0.2	-	-
	MS	-	0.5	0.3	0.3	-	-	-
OS1-5-1-1	Bfl	-	0.4	0.2	0.2	-	-	-
M. s.	B	-	0.6	-	0.2	0.3	-	-
	MS	-	0.5	0.1	0.2	0.1	-	-
OS1-5-1-2	Bfl	-	0.5	0.1	-	0.1	-	-
M. s.	B	-	-	-	0.1	0.2	-	-
	MS	-	0.2	0.5	0.4	0.3	-	0.2
OS1-9-1	Bfl	-	0.1	0.3	0.3	0.2	-	0.3
M. s.	B	-	-	-	-	-	-	-
	MS	-	0.3	0.5	0.1	-	-	-
OS1-10-1	Bfl	-	0.2	0.2	0.2	-	-	-
Phialophora sp.	B	-	-	0.4	0.6	-	-	-
	MS	-	-	0.2	0.2	-	-	-
OS1-14-1	Bfl	-	-	0.1	0.2	-	-	-
M. s.	B	-	-	0.1	0.1	-	-	-
	MS	-	0.4	0.1	0.2	-	-	-
OS1-15-1	Bfl	-	0.1	0.1	0.2	-	-	-
Phoma sp.	B	-	0.3	-	-	-	-	-
	MS	-	-	-	-	-	-	-
OS1-19	Bfl	-	-	0.2	0.1	0.2	-	-
M. s.	B	-	-	0.2	-	-	-	-
	MS	-	-	0.3	-	-	-	-
OS2-3-2-1	Bfl	-	-	0.2	0.2	-	-	-
Paecilomyces sp.	B	-	0.3	-	-	-	-	-
	MS	-	0.2	-	-	0.1	-	-
OS3-9	Bfl	-	0.2	0.2	0.2	-	-	-
Exophiala sp.	B	-	-	-	-	-	-	-
	MS	-	-	-	-	-	-	-
OS3-14	Bfl	-	-	0.2	0.2	-	-	-
M. s.	B	-	-	0.1	-	-	-	0.2
	MS	-	-	0.2	0.2	-	-	0.2
OS3-15-1	Bfl	-	-	0.1	-	-	-	-
M. s.	B	-	-	0.3	0.2	-	-	-
	MS	-	-	0.3	0.3	-	-	-
OS3-17-1	Bfl	-	-	-	-	-	-	-
Paecilomyces sp.	B	-	0.4	0.2	-	-	-	-
	MS	-	0.3	-	-	-	-	-
OS3-19	Bfl	-	0.3	0.2	0.3	-	-	-

Table 19 continued.

Fungal strain	Me-di-um	Inhibition (cm) against test organism						
		E. c.	***B. m.***	***M. v.***	***E. r.***	***F. o.***	***M. m.***	***C. f.***
Scopulariopsis sp.	B	-	0.1	0.1	-	-	-	0.3
	MS	-	0.3	0.5	-	-	-	-
OS4-2-1	Bfl	-	0.1	0.2	-	-	-	0.5
M. s.	B	-	0.2	0.3	0.1	-	-	-
	MS	-	-	-	-	-	-	-
OS4-2-2-2	Bfl	-	0.2	0.1	-	-	-	-
M. s.	B	-	-	0.3	-	-	-	-
	MS	-	-	-	-	-	-	-
OS4-2-3-1	Bfl	-	-	0.1	0.2	-	-	-
M. s.	B	-	-	-	-	-	-	-
	MS	-	-	0.8	0.3	-	-	-
OS4-2-3-2	Bfl	-	-	-	-	-	-	-
M. s.	B	-	-	-	0.2	-	-	-
	MS	-	-	-	0.3	-	-	-
OS4-3-1	Bfl	-	-	-	0.2	-	-	-
Chaetomium sp.	B	-	0.3	0.2	0.4	-	-	-
	MS	-	0.2	-	-	-	-	-
OS4-7-1	Bfl	-	-	-	0.1	-	-	-
Gliocladium sp.	B	0.1	0.3	0.5	0.4	-	0.1	-
	MS	0.1	-	1.0	0.3	-	-	-
OS4-12-1	Bfl	-	-	0.2	0.2	-	-	-
Wardomyces	B	-	0.1	-	0.3	-	-	0.2
anomalus	MS	-	-	0.3	0.3	-	-	-
OS4T3-2-1	Bfl	-	-	0.2	0.1	-	-	-
M. s.	B	-	0.1	0.2	0.1	-	-	-
	MS	-	-	0.3	0.1	-	-	-
OS4T3-2-2	Bfl	-	-	0.2	-	-	-	-
M. s.	B	-	-	0.4	-	-	-	-
	MS	-	-	0.4	-	-	-	-
OS5T2-1	Bfl	-	-	0.4	-	-	-	-
M. s.	B	-	-	0.2	-	-	-	-
	MS	-	-	0.2	-	-	-	-
OS5-2-1	Bfl	-	-	0.2	-	-	-	-
Chaetomium sp.	B	-	-	0.1	-	-	-	-
	MS	-	-	0.2	-	-	-	-
OS5-17-1	Bfl	-	-	0.3	-	-	-	-
Myrioconium sp.	B	-	0.2	0.6	0.9	-	-	-
	MS	-	0.3	1.5	1.2	-	-	-
OS5-19-1	Bfl	-	-	0.2	0.1	-	-	-

Table 19 continued: Results of the agar diffusion assays with EtOAc extracts of fungal cultures:

abbreviations and details.

Extracts were prepared, and agar diffusion assays were performed as described in the materials and methods section. Applied amounts were 250 µg extract per test disk. Inhibition zones (cm) were measured from the edge of the filter disks.

Tested strains:
Genus is given, and where available species name, M. s. = Mycelia sterilia.
The code marks the source sample (alga, sponge, jellyfish or plant) from which the fungal isolate was obtained, e.g., N1 is the sample *Fucus vesiculosus*. The numbering is an internal laboratory code. For origin of samples see 3.3.

Medium: B = solid biomalt medium; MS = solid malt extract Soya meal medium with 80 % ASW; Bfl = liquid biomalt medium with 100 % ASW; for media compositions see materials and methods section.

Test organisms: *E. c. Escherichia coli*, *B. m. Bacillus megaterium*, *M. v. Microbotryum violaceum*, *E. r. Eurotium repens*, *F. o. Fusarium oxysporum*, *M. m. Mycotypha microspora*, *C. f. Chlorella fusca*.

- no inhibition observed
TI total inhibition (over the whole agar plate)

Curriculum vitae

geboren:	14.8.71	in Helmstedt
Eltern:		Dr.-Ing. Rainer Wegner
		Doris Wegner, geb. Schultze
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	1984 - 1987	Gymnasium im Schulzentrum Rockwinkeler Landstr., Bremen
	1987 - 1990	Gymnasium an der Hermann-Böse-Str., Bremen
	1990	Abitur
Studium:	1990 - 1995	Pharmaziestudium an der TU Braunschweig
	3.7.96	Approbation als Apothekerin
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	1995 - 1996	Solvay Pharmaceuticals GmbH, Hannover
Weiterbildung:	29.10.99	Prüfung zum Fachapotheker für pharmazeutische Analytik
Berufstätigkeit:	1996-2000	Wissenschaftliche Mitarbeiterin am Institut für Pharmazeutische Biologie der TU Braunschweig
	seit 1.6.2000	Apothekerin in der Qualitätskontrolle bei Salutas Pharma GmbH, Magdeburg